National Library of Canada
Bibliothèque nationale du Canada

Canadian Theses Service
Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

NL-339 (r. 88/04) c

Canada

UNIVERSITY OF ALBERTA

THE INTERACTION OF PARAMYXOVIRUS MEMBRANE PROTEIN WITH ACTIN: A NOVEL MODEL TO STUDY VIRUS-INDUCED AUTOIMMUNITY

BY

WILAI TAECHAUDOMTAVORN

(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

FALL, 1990

National Library of Canada
Bibliothèque nationale du Canada

Canadian Theses Service
Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-64853-8

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: WILAI TAECHAUDOMTAVORN

TITLE OF THESIS: The INTERACTION OF PARAMYXOVIRUS MEMBRANE PROTEIN WITH ACTIN: A NOVEL MODEL TO STUDY VIRUS-INDUCED AUTOIMMUNITY

DEGREE: DOCTOR OF PHILOSOPHY

YEAR THIS DEGREE GRABTED: FALL 1990

PERMISSION IS HEREBY GRANTED TO THE UNIVERSITY OF ALBERTA TO REPRODUCE SINGLE COPIES OF THIS THESIS AND TO LEND OR SELL SUCH COPIES FOR PRIVATE, SCHOLARLY OR SCIENTIFIC RESEARCH PURPOSES ONLY.

THE AUTHOR RESERVES OTHER PUBLICATION RIGHTS, AND NEITHER THE THESIS NOR EXTENSIVE EXTRACTS FROM IT MAY BE PRINTED OR OTHERWISE REPRODUCED WITHOUT THE AUTHOR'S WRITTEN PERMISSION.

W. Taechaudomtavorn
(Student's Signature)

29/33 SOI SAENGCHAN,
(Student's Permanent Address)
RAMA 4 ROAD, PRAKANONG,
BANGKOK, 10110, THAILAND

Date: JUNE 1, 1990

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

THE UNDERSIGNED CERTIFY THEY HAVE READ, AND RECOMMEND TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH FOR ACCEPTANCE, A THESIS ENTITLED

THE INTERACTION OF PARAMYXOVIRUS MEMBRANE PROTEIN WITH ACTIN: A NOVEL MODEL TO STUDY VIRUS-INDUCED AUTOIMMUNITY

SUBMITTED BY WILAI TAECHAUDOMTAVORN

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN BIOCHEMISTRY

David L.J. Tyrrell

(DR. D.L.J. TYRRELL)

(DR. C. KAY)

(DR. D.G. SCRABA)

(DR. B. SINGH)

(DR. M. PEEPLES)

Date: JUNE 1, 1990

To My Parents

and

To My Husband, Chachaval Anomasiri.

ABSTRACT

The role of viruses in triggering autoimmune diseases is accepted, but the exact mechanism of viral-induced autoimmune responses often remains unclear. Actin is a conserved molecule and a weak antigen. Following some viral illnesses, anti-actin antibodies are detected in sera. In this thesis I have examined the interaction of a viral protein, the membrane protein of paramyxoviruses, with actin and the immunological consequences of this interaction.

The production of anti-actin antibodies is controlled by both genetic and environmental factors. B10 background major histocompatibility complex (MHC) congenic mice were used to examine for the genetic control of anti-actin antibody production. Mice which carry k alleles at the K locus of the MHC class I region produced antibodies when immunized with actin, whereas mice which carry other alleles at this locus did not produce antibodies under similar immunization conditions. This suggests that anti-actin antibody production is controlled by MHC class I genes. MHC class II genes had no effect on the antibody production to native actin.

When mice were immunized with a mixture of actin and M protein, all strains of mice studied produced anti-actin antibodies. To investigate the role of M protein on the production of anti-actin antibodies, the effect of M protein

on T-helper cell activation was studied. The *in vitro* response of T-cells to actin taken from animals immunized with actin plus M protein was unchanged from the T-cell response in animals immunized with actin alone. These studies were done in B10.BR and C57Bl/10 mice. In animals immunized with actin, the T-cell response to actin was significantly higher in B10.BR mice than in C57Bl/10 mice. Therefore, the addition of M protein to actin augments the antibody production in low responders, but not through the augmentation of T-cells specific for native actin.

Newcastle disease virus (NDV) M protein specifically binds to cellular actin as determined by affinity chromatography and enzyme linked immunosorbent assay. The interaction of M protein with actin resulted in antibody production to several new antigenic sites on the actin molecule. By immunoblotting with proteolytic fragments of actin, the new antigenic sites were located between amino acid residues 40-113, 114-226 and 227-375. The interaction of paramyxovirus M protein with actin and the subsequent production of antibodies to new antigenic sites may serve as a model for the study of virus-induced autoimmunity.

ACKNOWLEDGEMENTS

Firstly I would like to express my deep appreciation for the support of my supervisor Dr. D.L.J. Tyrrell. He has been especially friendly and helpful. I am grateful for his time, interest, advice and guidence throughout the course of these studies.

I would like to extend my gratitude to my supervisory committee including Drs. D. Scraba, W. Bridger and B. Singh for their kindness and advice.

I wish to thank Drs. G. McFadden, G.D.A. Armstrong for the use of their facillities and their generous guidance and enthusiasm.

I would like to thank Dr. D.R. Tovell and Mrs. Linda Chui for all the technical assistance throughout the years, together with all of the members of Dr. Tyrrell's laboratory, the Department of Biochemistry and the Department of Medical Microbiology and Infectious Diseases who have made my time in Edmonton memorable.

The financial support of the Department of Biochemistry and the Medical Research Council of Canada during this project is gratefully acknowledged.

Finally I would like to acknowledge the continuous interest and encouragement of my husband, Chaohaval Anomasiri, as well as the moral support of my parents and the Taechaudomtavorn family.

TABLE OF CONTENTS

CHAPTER		PAGE
1.	Introduction	1
	Bibliography	38
2.	Interaction of Actin and Paramyxovirus M Protein	48
	Bibliography	71
3.	Paramyxovirus M Protein Augments the Production of Anti-actin Antibodies	73
	Bibliography	99
4.	Mechanism of Virus M Protein Induced Autoimmunity to Actin	102
	Bibliography	125
5.	Effect of the Expression of the Cloned M Gene on Actin Microfilaments in Fibroblasts	128
	Bibliography	153
6.	General Conclusions and Future Prospects	156
	Bibliography	163

LIST OF TABLES

Table	Description	Page
1.1	Classification of paramyxoviridae	35
2.1	Actin monomer sequestering proteins	65
2.2	Competitive binding of ^{125}I-actin to virus M protein coated on polystyrene tubes	66
3.1	Enhancement of antibody production in Balb/c mice by NDV M protein	90
3.2	Immune response to actin alone and actin and NDV M protein in Balb/c and Balb/c (nude) mice	91
4.1	Properties of class I and class II molecules	120
4.2	Genetic control of immune response to actin using actin primed and actin mixed with M protein primed mice	121

LIST OF FIGURES

Figure	Description	Page
1.1	Predicted amino acid sequence of M protein of measles virus	36
1.2	Amino-terminal sequence of actin isoforms	37
2.1	SDS-PAGE of protein applied to and eluted from an affinity column of NDV M protein linked to Sepharose 4B	67
2.2	Interaction of M protein and actin as determined by ELISA	68
2.3	Scatchard analysis of actin-M protein binding data	69
2.4	Scatchard analysis of carbonic anhydrase-M protein binding data	70
3.1	Effect of M protein and DNase-I on actin antibody production	92
3.2	Effect of M protein on carbonic anhydrase antibody production	93
3.3	Actin fragments visible in SDS-PAGE gels after protease digestion	94
3.4	Antigenic mapping	95
3.5	Western blotting results for human antisera containing measles antibodies and/or anti-actin antibodies.	96

Figure	Description	Page
3.6	The membrane protein of NDV and anti-actin antibodies bind to different sites on actin.	97
3.7	ELISA determination of M protein binding to N fragment of actin cleaved by *S. aureus* V-8 protease	98
4.1	Comparative studies of immune response to actin from T cells of B10.BR mice and C57Bl/10 mice in actin immunized mice and actin plus M protein immunized mice	122
4.2	Effect of monoclonal antibodies to MHC antigen on T cell proliferation assay	123
4.3	Carrier effect of M protein on anti-actin antibody production	124
5.1	Genetic maps of plasmid DNA used in this study	147
5.2	Agarose gel electrophoresis of plasmid DNA digested with restriction endonuclease enzymes, HindIII and SstI to confirm the insertion of cloned M gene	148
5.3	Plasmid DNA digested with restriction endonuclease enzymes, SalI or BglII	149
5.4	Pathway of purine nucleotide synthesis and the inhibition sits of aminopterin and mycophenolic acid	150

Figure	Description	Page
5.5	Double labelled immunofluorescent staining of measles virus-infected CV-1 cells.	151
5.6	Double labelled immunofluorescent staining of pMR transfected NIH 3T3 cells.	152

LIST OF ABBREVIATIONS

ABTS	2,2'-azino, di [3-ethyl-benzthiazoline sulfonate]
APCs	antigen presenting cells
ATCC	American Type Culture Collection
BFA	brefeldin-A
BSA	bovine serum albumin
cDNA	complemetary DNA
CFA	complete Freund's adjuvant
CGN	chronic glomerulonephritis
Ci	Curies
CNB	cyanogen bromide
con A	concanavalin A
CsCl	cesium chloride
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
DNaseI	deoxyribonuclease I
EBV	Epstein-Barr virus
EDTA	disodium ethylenediaminetetra acetate
ELISA	enzyme-linked immunosorbent assay

ER	endoplasmic reticulum
F protein	fusion protein
Fig.	figure
GAT	L-glutamine-L-alanine-L-tyrosine
GMP	guanosine monophosphate
gpt	*E. coli* xanthine-guanine phosphoribosyltransferase
h	hour
HA	hemagglutinin
HBsAg	hepatitis B surface antigen
HLA	human leukocyte antigen
HN	hemagglutinin and neuraminidase
HPLC	high performance liquid chromatography
IC_{50}	concentration resulting in 50% inhibition
IDDM	insulin dependent diabetes mellitus
IFA	incomplete Freund's adjuvant
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
IMP	inosine monophosphate
Ka	association constant
kb	kilobases

KCl	potassium chloride
K_D	dissociation constant
kDa	kilodaltons
KLH	keyhole limpets hemocyanin
L protein	large protein of Paramyxoviruses
LB medium	Luria-Bertani medium
LTR	long terminal repeat
M protein	membrane protein of paramyxoviruses
MCMV	murine cytomegalovirus
MHC	major histocompatibility complex
min	minute
ml	milliliter
mm	millimeter
mmole	millimole
MMTV	mouse mammary tumor virus
MOI	multiplicity of infection
mRNA	messenger RNA
MW	molecular weight
n	number
N.S.	not significant
NDV	Newcastle disease virus

nm	nanometer
NMR	nuclear magnetic resonance
NP	nucleoprotein of paramyxoviruses
°C	degree Celsius
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
PPD	purified protein derivative
ρ	density (g/ml)
RNA	ribonucleic acid
RNase-A	ribonuclease A
rpm	revolutions per minute
RSV	respiratory syncytial virus
S aureus	*Staphylococcus aureus*
S.D.	standard deviation
SDS	sodium dodecyl sulfate
SLE	systemic lupus erythematosus
SSPE	subacute sclerosing panencephalitis
SV	simian virus
TE buffer	10 mM Tris, 1 mM EDTA, pH 8.0
Th	helper T cells

ts	temperature sensitive
VSV	vesicular stomatitis virus
XGPRT	xanthine-guanine phosphoribosyltransferase
µg	micrograms
µl	microliters
µM	micromolar

CHAPTER 1

Introduction

Viruses are obligate intracellular parasites that depend on cellular metabolism for their reproduction. They can initiate disease by different mechanisms including viral-induced cytotoxicity or immune-mediated host response. A successful immune response to viral infection involves a complex cascade of events, including presentation of viral antigens to the immune system, proliferation of antigen-specific lymphocytes, and production of an array of lymphokines. This expansion phase leads to an effector phase that eliminates virus from blood and tissue and provides long-term immunity for many common viruses such as measles or mumps. In rare instances, the viruses are not completely eliminated by the body's immune system and slow or persistent infections result. Viruses in persistent infections in particular, have a considerable potential to cause or trigger autoimmune phenomena. However, there are very few situations where viruses can be directly incriminated as causative agents of autoimmune diseases. Over the past decade and with the advent of new technologies, evidence is rapidly accumulating to support the existence of possible roles for both RNA and DNA viruses in autoimmune diseases (Rager-Zisman, 1988). However, the mechanisms by which a viral

infection can lead to the production of autoimmune responses are poorly understood.

Autoantibodies arising as a consequence of viral infections have been described previously, and in most experimental systems they are short lived and of low titer (Notkins, *et al.*, 1984). Multiple autoantibodies in the sera of mice infected with murine cytomegalovirus show complex patterns of intraorgan, intratissue and intracellular reactivity (Bartholomaeus, *et al.*, 1988). For example, autoantibodies reacted with the kidney cells lining the tubule but not to cells of the glomeruli. Anti-nuclear antibodies were detected in the sera of mice as early as seven days after virus infections.

In humans, autoantibodies to cells and tissue antigens have been detected after viral infections. Smooth muscle antibodies that react with actin are often detected in the sera of patients following acute generalized viral diseases, such as hepatitis A (Farrow, *et al.*, 1970), infectious mononucleosis (Holborow, *et al.*, 1973), mumps and measles (Haire, 1972). The mechanism by which these viruses induce antibodies to actin is not clear. Fagraeus *et al.* (1983) produced autoantibodies to actin and vimentin in Balb/c mice immunized with paramyxoviruses. Giuffre *et al.* (1982) had revealed that paramyxovirus membrane/matrix (M) protein was able to react with actin. This specific interaction of a viral protein with a host cell protein and the subsequent

production of autoantibodies to the host cell protein suggests a specific role for the viral protein in this response. The interaction of M protein with actin and the mechanism by which this viral protein induces an autoimmune response to actin is the principal subject of this thesis.

Paramyxovirus

The *paramyxoviridae* family is a large group of viruses divided into three genera according to their biological and biochemical characteristics, with particular reference to the properties of the attachment proteins (Mathews, 1982) (Table 1). The three genera are the paramyxoviruses, the morbilliviruses, and the pneumoviruses. Members of the paramyxovirus subgroup exhibit both neuraminidase and hemagglutinating activities (Choppin and Compans, 1975). Morbilliviruses differ from the paramyxoviruses in that they lack neuraminidase activity. The pneumoviruses have neither hemagglutinating nor neuraminidase activities (Gruber and Levine, 1983).

Virus structure

The virion of *paramyxoviridae* consists of a ribonucleoprotein core surrounded by a host cell-derived lipid envelope. The virus genome is a linear, non-segmented,

single-stranded, negative-sense RNA molecule. It is never infectious by itself; it does not act as a messenger for viral proteins, but it needs virus-specified RNA polymerase proteins to transcribe its messenger RNAs which are complementary to the virion RNA (Baltimore, 1971). The replication of a paramyxovirus as demonstrated in lymphocytes does not require DNA synthesis (Lucas *et al.*, 1979). Primary transcription can occur in the presence of inhibitors of DNA or protein synthesis because the infecting virus carries its own RNA polymerase. The genome of paramyxovirus is 15 to 18 kb long and contains at least six genes (Dubois-Dalcq *et al.*, 1984). The order of the genes in measles virus genomic RNA is 3'-NP, P+C, M, F, HA, L-5' (Richardson *et al.*, 1985).

Paramyxovirus particles are pleomorphic, and range in size from 300 to 1000 nm in diameter with large surface glycoprotein projections. The helical nucleocapsid, coiled within the particle, has a diameter of 21 nm (Lund *et al.*, 1984). The structure and function of *paramyxoviridae* proteins can be summarized as follows:

1. **L protein** (MW 150-200 kDa). The L protein is present in small amounts in the virion and is associated with nucleocapsids and likely involved in the enzymatic functions associated with synthesis and modification of viral mRNA (Choppin and Compans, 1975).

2. **HA** (MW 65-74 kDa). The HA protein is the attachment protein. A comparison of the sequences and the structures of the major glycoprotein such as position of the cysteine residues, the glycosylation sites and the hydrophobic domains of the seven attachment proteins of *paramyxoviridae* supports the division of the members of the family into three groups (Morrison, 1988). The position of the membrane-spanning domain near the amino terminus of the HA proteins is the only feature of these proteins that appears to be conserved across the family.

3. **P protein** (MW 70-79 kDa). The P protein is a phosphorylated protein which is associated with the nucleocapsids and may function in either transcription or replication. One striking and unusual feature of the P genes of most paramyxoviruses is the bicistronic nature of the P messenger RNA. The P protein mRNA encodes both the P and C proteins (Giorgi *et al.*, 1983; Bellini *et al.*, 1985).

4. **N protein** (NP) (MW: 56-60 kDa). The N protein is the major structural protein of the nucleocapsid and the most abundant protein in the virion. It binds tightly to the virion RNA, protecting it against nucleases.

5. **Fo protein** (MW 65-68 kDa). The fusion proteins of all paramyxoviruses are synthesized as a precursor, Fo, which is subsequently cleaved to the biologically active form, a disulfide bond-linked heterodimer composed of F1 (MW 47-56

kDa) and F2 (MW 10-16 kDa) (Scheid and Choppin, 1977). The F protein is responsible for cell fusion and hemolysis.

6. **M protein** (MW 34-40 kDa). The membrane or matrix (M) protein is a nonglycosylated protein with hydrophobic properties. It plays a key role in assembly and budding, and is the viral protein most likely to be defective in persistent measles virus infections (Hall *et al.*, 1979; ter Meulen *et al.*, 1983).

7. **C protein** (MW 22 kDa). It is a nonstructural protein found in infected cells and in cell free translation products, but the role of C protein in infection is unclear (Bellini *et al.*, 1985).

In addition to virus-coded proteins, cellular actin (MW 43 kDa) has been identified in paramyxoviruses including Sendai virus (Lamb *et al.*, 1976) and measles virus (Tyrrell and Norrby, 1978). The possible role of actin in the assembly of the virus will be discussed later.

Structure and function of M protein

Analyses of the deduced amino acid sequence has been carried out for the M proteins of Sendai, measles, canine distemper viruses and Newcastle disease virus. These studies reveal that M protein is highly basic (Blumberg *et al.*, 1984; Bellini *et al.*, 1986; Chambers *et al.*, 1986). A hydropathic

profile of the M protein amino acid sequence suggests that, although the M protein is hydrophobic in character, there are no sections of the sequence of sufficient length and hydrophobicity to span the cell membrane (Kyte and Doolittle, 1982). The amino acid homology among the *paramyxoviridae* M proteins shows that the sequences of M proteins of measles and canine distemper virus (76.1%) and the sequences of the M proteins of Sendai virus and parainfluenza virus type 3 (62.1%) are highly conserved. Moderate homology is seen in the sequences of the M protein of measles virus versus Sendai virus (36.8%). The M proteins of mumps virus and Newcastle disease virus show only 23-27% homology with M proteins of other paramyxoviruses (Elango, 1989). The secondary structure of Sendai virus M protein is 11% α-helix, 19% β-pleated sheet, and 70% random coil based upon circular dichroism measurements (Giuffre *et al.*, 1982).

M protein is the nonglycosylated protein in paramyxovirus envelopes. It is phosphorylated in Sendai virus (Lamb and Choppin, 1977) while a nonphosphorylated form is found in measles virus (Wong *et al.*, 1987). The phosphorylated form of M protein may regulate virus replication and/or transcription while the non-phosphorylated form may be involved in virion formation (Yoshida *et al.*, 1976).

There are numerous activities attributed to the M protein of *paramyxoviridae*. The M protein accumulates on the

inner surface of the lipid bilayer of infected cells, where it interacts with the cytoplasmic domains of the two integral viral glycoproteins (HA and F proteins) (Choppin and Compans, 1975). It constitutes an intermediary structure between the internal core and the outer envelope (Norrby, 1985). M protein appears to be involved in mediating the association of viral proteins with the plasma membrane. It is through the interactions of M protein with nucleocapsids and the viral glycoproteins in the cell membrane that assembly of progeny virus and budding from the plasma membrane is thought to occur (Yoshida *et al.*, 1976; Tyrrell and Ehrnst, 1979; Tuffereau and Roux, 1988). Sendai virus M protein self-assembles into an ordered structure (Heggeness *et al.*, 1982), and interacts with cellular actin (Damsky *et al.*, 1977; Giuffre *et al.*, 1982). The Newcastle disease virus M protein has also been found in the infected cell nucleus but its function in the nucleus is unknown (Peeples, 1988; Faaberg and Peeples, 1988).

In persistently infected cells, defects in M protein have been demonstrated for Sendai virus and measles virus (Cattaneo *et al.*, 1986; Tuffereau and Roux, 1988). A large number of studies have shown an association of measles virus with subacute sclerosing panencephalitis (SSPE). Most SSPE patients have been shown to have reduced levels of M protein antibodies (Hall *et al.*, 1979). In the review by ter Meulen and Carter (1982), the authors suggest that the defect of

measles virus replication in SSPE brain tissue may be due to a defect in the transcription or the translation of mRNA for M protein. Comparison of the M protein mRNA expression in measles-infected cells and SSPE virus-infected brain tissue indicated that there was impairment of measles virus M protein mRNA synthesis in infected brain tissue (Baczko *et al.*, 1984). Genetic analysis of the M protein coding sequence from a virus isolated from a SSPE patient showed that there was a collection of point mutations in the coding region for M protein. This genetic defect may explain the lack of viral M protein in SSPE brain cells (Cattaneo *et al.*, 1986). Recently, the nucleotide sequence of the M genes of three SSPE virus strains Niigata-1, ZH, and Biken, have been studied in order to explain the defective M-mRNA and M protein content. The results showed that there was premature termination of M-mRNA in Niigata-1 and ZH strains, and there was rapid degradation of the M protein synthesized by the Biken strain (Enami *et al.*, 1989). The association of defectiveness of SSPE virus with the instability of the M protein or M-mRNA supports the concept that M protein plays a major role in the maturation of the virus. Defects in M protein expression alter virion assembly and may lead to persistent virus infections with spread from cell to cell by fusion rather than the production of budding virus and subsequent acute infection of cells at distant sites.

The role of the cytoskeleton in virus budding

The identification of the cytoskeletal protein, actin, in many enveloped viruses (Wang *et al.*, 1976) including measles virus (Tyrrell and Norrby, 1978), Sendai virus (Lamb *et al.*, 1976) and Newcastle disease virus (Morrison and McGinnis, 1985) raises the question of the role of actin in the structure and/or replication of these viruses. Little is known about virus and cytoskeletal interactions, although previous studies from our laboratory have implicated the cytoskeleton as having an important role in the transport of viral proteins to sites of assembly, and the assembly and transport of viral nucleocapsids to sites of virion maturation and release (Tyrrell and Ehrnst, 1979). Studies of interactions between cytoskeletal elements and viral proteins may help us to understand the mechanisms of virus maturation and the role of the cytoskeleton in virus budding from infected cells.

Virus assembly consists of a series of events governed by interactions of virus proteins with each other and with the virus-modified membrane at the budding site. All the paramyxovirus proteins are synthesized and processed in the cytoplasm. The glycoproteins, HA and F, are synthesized in the rough endoplasmic reticulum (ER). They begin with an N terminal leader sequence, and are directly inserted into the ER membrane. They are processed in the Golgi apparatus prior to being transported to the infected cell surface. The other

proteins (N, L, P), which associate with the genome to form nucleocapsids, are synthesized on free ribosomes. M protein is also synthesized on the free ribosomes, but is very quickly bound at the plasma membrane in areas where viral glycoproteins are present. It is assumed that the predominant interaction between M protein and the membrane takes place between the cytoplasmic domains of the viral glycoproteins and the M protein. During virus budding, paramyxovirus nucleocapsids move to an area of the cell membrane rich in M protein and glycoprotein before being enveloped (Choppin and Compans, 1975). Transmembrane interaction between surface-associated envelope components and the intracellular measles virus matrix and nucleocapsid proteins is demonstrated by co-capping of the intracellular components by antibodies against the surface glycoproteins (Tyrrell and Ehrnst, 1979). These observations supported the concept that the M protein functions as a bridge between the envelope and the nucleocapsids. The inhibition of co-capping of the intracellular proteins and the surface glycoproteins in the presence of cytochalasin B suggested that the cytoskeleton played a role in the movement of intracellular components to the site of viral budding.

Results from the deduced amino acid sequence of Newcastle disease virus (NDV) M protein show that it contains 14% basic and 8% acidic amino acids. The M proteins of Sendai virus and canine distemper virus are also very basic.

The net positive charge of M protein (pI of vesicular stomatitis virus M protein is 9.1) (Zakowski *et al.*, 1981) may play a role in its strong association with actin since actin has an overall negative charge (pI=5.4) (Garrel and Gibson, 1976). The presence of actin in enveloped viruses may reflect a role of the cytoskeleton in virus assembly.

The role of actin microfilaments in the movement of virus components to the plasma membrane and their role in the process of budding still remain unclear. Cells infected with paramyxoviruses such as measles or mumps, often demonstrate a decrease in the number of actin filament bundles as shown by indirect immunofluorescence (Fagraeus *et al.*, 1978). The rapid decrease in microfilament bundles was also observed in vaccinia virus-infected fibroblasts (Meyer *et al.*, 1981). This study also showed that in the presence of puromycin, an inhibitor of viral protein synthesis, the microfilament bundles of infected cells were significantly preserved. These observations suggest that cellular microfilaments are disrupted by the synthesis of a specific viral protein. Using phenothiazines, Bohn *et al.* (1983) demonstrated a correlation between the formation of viral buds and the presence of cellular microvilli, suggesting that both structures are synthesized under similar circumstances. Cytoskeletal filaments are associated with the M protein of Newcastle disease virus and measles virus as well as the

other viral proteins as demonstrated by detergent extraction procedures (Bohn *et al.*, 1986; Morrison and McGinnis, 1985).

The involvement of cytoskeletal elements in paramyxovirus assembly has also been inferred from data obtained by studying the effects of inhibitors of cytoskeleton activity, such as cytochalasin B and cytochalasin D. These compounds bind to the barbed end of actin filaments; this inhibits both the association and dissociation of subunits from that end. Thus, the binding of either cytochalasin to actin inhibits polymerization and depolymerization of actin. In addition to binding to actin, cytochalasin B also inhibits monosaccharide transport across the plasma membrane, whereas cytochalasin D does not (Cooper, 1987). In measles virus-infected Vero cells, cytochalasin B causes the accumulation of viruses on the cell surface, and release of the viral particles is drastically decreased (Stallcup *et al.*, 1983). The cytochalasin B inhibition of cellular functions is reversible and, similarly, the inhibition of virus release was completely reversed upon removal of the drug. This inhibition was also demonstrated with cytochalasin D, which disrupts microfilaments without affecting protein glycosylation. Thus, it was concluded that alterations in microfilament structure can affect the normal budding process of measles virions. These results favor the hypothesis that the forces resulting from the vectorial growth of actin filaments contribute to the formation of

viral buds and that actin appears to play a major role in virus budding.

However, the view that actin filaments are involved in virus budding is not universally accepted. Griffin and Compans (1979) found that cytochalasin B inhibited the release of influenza virus and deduced that the inhibition was secondary to an inhibition of glycosylation because the inhibition of virus release was reversed by the addition of exogenous neuraminidase. Treatment of NDV-infected cells with cytochalasin D suggests that functional microfilaments do not play a role in viral morphogenesis but they function to slow the release of viral particles from the cells (Morrison and McGinnes, 1985).

Actin structure

Actin is present in all living cells. A comparative study of the amino acid sequence of actin from five different phyla of invertebrate organisms and warm-blooded vertebrates has been completed in order to study the molecular evolution of muscle-specific actins (Vandekerckhove *et al.*, 1983). In invertebrates, there is only one major muscle actin form, and it is homologous to the actins of chordate and invertebrate non-muscle cells. The number of different muscle actin types increases from one in lower vertebrates to two or three in amphibia to four in warm-blooded vertebrates. Their

expression is controlled in a tissue-specific manner. Vertebrate actins are categorized into three groups (alpha, beta, and gamma) on the basis of separation using isoelectric focusing (Garrel and Gibson, 1976). At least six actin isoforms are expressed in mammals and birds as characterized by two-dimension polyacrylamide gel electrophoresis and amino acid sequence analysis (Vandekerckhove and Weber, 1978): two sarcomic (cardiac muscle α actin and skeletal muscle α actin), two smooth muscle (vascular α actin and non-vascular γ actin) and two cytoplasmic actins (non-muscle β and γ actins) (Bulinski *et al.*, 1983). These actins show a high degree of homology in their primary structure except for the N-terminal region. The N-terminal variability serves as the basis for the isoactin classification (Vandekerckhove and Weber, 1984). Actin isoforms show only 50-80% sequence homology in the 18 residues at their N-termini, each isoform having a unique sequence (Vandekerckhove and Weber, 1978). In all cases, however, the terminal 17 amino acids are characterized by a cluster of negative charges (three or four consecutive dicarboxylic amino acid residues) (Vandekerckhove and Weber, 1984). The presence of many acidic amino acids in this region of actin accounts for its low isoelectric point. Most of the substitutions in actin isoforms are considered to be conservative amino acid replacements (Hightower and Meagher, 1986). In spite of this noticeable homology, actins exhibit some differences in their functional properties such as polymerization properties, filament stability, susceptibility

to DNaseI depolymerization, and susceptibility to shearing force (Hue *et al.*, 1988).

The N-terminal 17 amino acids of smooth muscle actins is the region where most of the sequence changes are concentrated. NMR studies have shown that the N-terminal region is the most mobile segment of the actin molecule (Barden and dos Remedios, 1983). It is also one of the most hydrophilic regions of the molecule (Roustan *et al.*, 1986). These two characteristics, mobility and hydrophilicity, are correlated with antigenicity (Van Regenmortel, 1986). Synthetic peptides as well as N-terminal tryptic peptides have been utilized to prepare isoform-specific anti-actin antibodies. These specific antibodies have been used to study the isoforms of actin in smooth muscle differentiation in normal and pathological conditions, to detect muscle-specific actin isoforms in differentiating mouse and human myoblasts, and to study the actin isoform distribution in cells and tissues. Roustan *et al.* (1986) reported the presence of two antigenic epitopes in skeletal-muscle actin, corresponding to the sequences embracing residues 1-7 and 18-28.

Paramyxovirus infection and autoimmune responses

One of the primary features of the immune system is self-non-self discrimination. Once the immune system can

distinguish between what is "self" and what is "not-self", "self" is preserved and "not-self" is destroyed. However, it is clear that the distinction between self and non-self is not absolute. Several autoimmune disorders, in which the immune system attacks normal, healthy tissue, have been described. The list of known or suspected autoimmune diseases has been expanded to include: systemic lupus erythematosus, in which DNA, blood vessels, skin and kidneys are attacked by the immune system; multiple sclerosis, in which the tissue attacked is myelin (a substance that sheaths nerves in the central nervous system); myasthenia gravis, in which the target is a receptor molecule for the neurotransmitter acetylcholine; rheumatoid arthritis, in which the target is primarily the peripheral joints; and type 1 (juvenile) diabetes mellitus, in which the cells producing insulin are destroyed by an immune response. Despite the growing list of autoimmune diseases, the mechanism by which these autoimmune responses are triggered remains unknown for most diseases.

Factors involved in autoimmunity

Shoenfeld and Isenberg (1989) have reported low levels of autoantibodies in a number of normal individuals. They have suggested that self-recognition may be normal in some physiological states and that autoantibodies do not always

imply a pathological condition. They also acknowledged that autoimmune diseases are multifactorial in origin and that when these factors are combined in different subjects in different ways, a distinct autoimmune disease may be induced. They have categorized factors involved in autoimmunity into five groups: (1) genetic, (2) immune defects, (3) hormonal, (4) environmental influences and (5) others.

Family studies of autoimmune diseases by Bias *et al.* (1986) showed that the familial occurrence of autoimmune traits, as defined by the presence of autoimmune disease and /or high titer autoantibody, supports the hypothesis that one of the factors in autoimmunity is inherited as an autosomal dominant trait. Most autoimmune conditions are associated with a specific major histocompatibility complex (MHC) antigen profile. In humans, this MHC antigen is called human leukocyte antigen (HLA), which is equivalent to the H-2 antigen in mice. The most common associations between HLA and autoimmune disorders have been identified with multiple sclerosis and HLA-DR2, celiac disease and HLA-DQw2, systemic lupus erythematosus and HLA-B8, juvenile rheumatoid arthritis and HLA-DR3 and DR4, ankylosing spondylitis and Reiter's disease and HLA-B27 (Cruse and Lewis, 1988). In addition, even though autoantibodies are considered important factors in the understanding of autoimmunity, there has been increasing evidence for a role for T cells in autoimmune diseases. Studies of autoimmunity in type 1 juvenile

diabetes mellitus (or insulin-dependent diabetes mellitus, IDDM) have demonstrated that autoantibodies, alteration of T lymphocyte activation, and linkage to the immune response genes of the HLA-D region are all features of IDDM and may precede clinical manifestation of this disease (Cruse and Lewis, 1988).

Environmental factors such as bacteria, parasites, drugs and viruses may be the inducing agents of autoimmunity in individuals with an appropriate "immuno-genetic-hormone" background. Rubella, mumps, and Coxsackie B viruses have been shown to be associated with IDDM (Doniach *et al.*, 1983). Coxsackie B3 virus infection has been found associated with autoimmune myocarditis (Rose *et al.*, 1988). Infection with C type virus may lead to the development of SLE. Hepatitis B antigens have been found in 25-40% of polyarteritis nodusa patients, which suggests that this virus may play a role in the etiology of this disease (Chapel and Haeney, 1984). Infections with paramyxoviruses such as mumps and measles viruses are associated with the production of autoantibodies to actin, although this response has not been associated with an autoimmune disease.

Immune responses to paramyxovirus infection

The natural result of infection is the induction of cellular and humoral immune responses to viral antigens in

order to eliminate the infectious agent before serious harm occurs. Virus clearance occurs coincidentally with the appearance of neutralizing antibodies directed to attachment epitopes on the virus and the production of cytotoxic T-cells. The neutralizing antibodies may interfere with the interaction between the virion and the cell surface receptor, macrophage opsonization, and may play a role in cell-mediated cytotoxicity by cytotoxic T cells or the elimination of virally infected cells by natural killer cells. However, until viral antigens are synthesized and expressed on the surface of infected target cells or until virions are present in sufficient quantity in the circulation, humoral immunity and the generation of cytotoxic T cells will not occur.

Measles is a well characterized acute viral infection of childhood. The disease has become more common in older teenagers or young adults in the post-vaccination era. The incubation period of acute measles is 10 to 14 days. A characteristic prodromal phase is marked by conjunctivitis, cough and the appearance of Koplik's spots. The rash marks the onset of the effector phase of the immune response, with the appearance of antibodies, infiltration of lymphocytes into areas of viral replication in the skin and initiation of viral clearance from infected tissues. Viral clearance is essentially complete in immunologically competent persons within five days. The levels of soluble CD8, a marker of effector-target cell interaction, are high during the

appearance of the rash, and return to normal soon after the disappearance of the rash (Moench *et al.*, 1988). Measles virus infection may be associated with an alteration in immune function. The disease may suppress cell-mediated immunity and promote the reactivation of tuberculosis in tuberculin-positive patients. In adddition, measles virus infections have been associated with secondary autoimmune responses. The most serious of these is postmeasles autoimmune encephalitis and demyelinating disease (Johnson, 1987).

Evidence for measles virus in autoimmune diseases.

Autoimmune diseases have quite commonly been associated with high titers of antibodies to viral antigens. For example, Epstein-Barr virus antibody is elevated in patients with SLE (Evans *et al.*, 1981). In many studies measles virus antibodies have been reported as elevated in patients with multiple sclerosis. I will not attempt to review this extensive literature as this association remains interesting but not conclusive.

The presence of high titers of antibodies against measles virus antigens in sera from patients with hepatitis B surface antigen (HBsAg)-negative chronic active hepatitis has been reported by Closs *et al.* (1971) and Christie and Haukenes (1982). The sera from these patients contain a

large amount of measles-specific IgG and IgM antibodies. The presence of virus-specific IgM antibodies may serve as an indicator for the persistence of the viruses. Thus, it is interesting to compare the titers of the measles virus IgM antibodies in sera from patients with an autoimmune disorder with the titers in sera from patients with acute measles virus infections. Sera from patients with chronic active hepatitis four), acute measles virus infection (two), infectious mononucleosis (two) and healthy controls (two) were examined for measles-specific IgM antibodies (Christie, *et al.*, 1984). IgM immune complexes were obtained from sera of all four patients with chronic active hepatitis and from the two patients with acute measles virus infection. These IgM immune complexes from patients with chronic active hepatitis or acute measles contained the measles virus membrane (M) protein and nucleoprotein (NP), There were only traces of the other viral antigens including hemagglutinin, fusion and phosphorylated proteins. There were no IgM antibodies against any of the measles virus polypeptides present in the sera of patients with infectious mononucleosis or in the sera of healthy individuals with a remote history of measles infection.

In addition, the measles virus genome has been found in lymphocytes from patients with autoimmune chronic active hepatitis (Robertson *et al.*, 1987). Whether measles virus plays an etiological role in the development of this form of

hepatitis remains controversial (Black, 1988). The significance of the presence of measles virus genome in lymphocytes in autoimmune chronic active hepatitis has also been questioned by Kalland, *et al.* (1989). Using cDNA synthesis and the polymerase chain reaction (PCR), they studied the total RNA from lymphocytes in eight patients with autoimmune chronic active hepatitis and six healthy controls. There was a band of the correct size from the PCR reaction which hybridized with the measles virus probe in seven of the eight patients and in four of the six controls. The amount of measles virus RNA in lymphocytes from patients with chronic active hepatitis was at least 5000 times lower than the amount of measles virus RNA in infected Vero cells. These results would suggest that measles virus does not play a significant role in chronic active hepatitis. Andjaparidze, *et al.* (1989) have also detected measles virus RNA in tissues from patients with systemic lupus erythematosus (SLE) or chronic glomerulonephritis (CGN). They have assayed for the presence of measles virus genomes in lymphocytes of peripheral blood from 34 patients with SLE and 68 patients with CGN, using dot-blot hybridization with a pBR322 plasmid containing the measles virus nucleoprotein gene. Viral RNA was detected in the lymphocytes of 28 of 34 (82%) patients with SLE and of 40 of 68 (59%) patients with CGN, but was not detected in the peripheral lymphocytes from 29 control patients. This report, if confirmed in follow-up

studies, would add support to the concept that measles virus may play a role in some chronic autoimmune diseases.

Anti-actin antibodies

Considerable difficulty has been encountered in producing polyclonal antibodies to undenatured actin, presumably because actin is a highly-conserved molecule (Fagraeus and Norberg, 1978). Reasonable anti-actin antibody titers have been produced in experimental animals only after considerable modification of the actin antigen. To date, most anti-actin antibodies have been produced in animals immunized with denatured actin. For example, antibodies have been produced to actin after treatment with sodium dodecyl sulfate (Lazarides and Weber, 1974; Nishioka *et al.*, 1986), insolubilization on alum (Jockusch *et al.*, 1978), chemical cross-linking and modification with glutaraldehyde (Roustan *et al.*, 1985; Benyamin *et al.*, 1986), incubation of G-actin at 4 °C for at least a week (Trenchev and Halborow, 1976), or incubation of G-actin at 60 °C for 10 minutes (Hambly *et al.*, 1983). These physicochemical treatments of the actin molecule are effective in modifying native actin to become a more potent antigen.

Autoantibodies to cytoskeletal components have frequently been detected in human and animal sera. Autoantibodies to smooth muscle antigens were first described

using an immunofluorescence technique on tissue sections (Johnson *et al.*, 1965). Later studies using the sera from patients with acute, generalized viral infections, such as cytomegalovirus infection (Andersen and Andersen, 1975), Epstein-Barr virus infection (Seigeneurin *et al.*, 1988), adenovirus and influenza B virus infections (Portocala *et al.*, 1988), human immunodeficiency virus infection (McClure and Shearer, 1988), and mumps and measles virus infections (Haire, 1972) demonstrated the presence of smooth muscle antibodies. The close relationship between smooth muscle antibodies and anti-actin antibodies has been confirmed by several immunological procedures (Gabbiani *et al.*, 1973). Monoclonal anti-actin antibodies were produced by hybridomas obtained from mice immunized with purified virions of paramyxoviruses which contained actin (Fagraeus *et al.*, 1983). In addition, monoclonal antibodies to rat brain actin were easily produced when mice were immunized with HVJ (Sendai virus) M protein to enhance the antigenicity of actin (Hiruma and Hashimoto, 1988).

It is not known what initiates the production of anti-actin antibodies *in vivo*. The studies listed above suggest that viral infections may play a role in the production of anti-actin antibodies. It is probable that the immunological tolerance to actin is broken as a result of cell damage induced by a virus (Nishioka *et al.*, 1983). McClure and Shearer (1988) have postulated that anti-actin antibody

production is triggered by a process of molecular mimicry in response to viral oncogenes or to the abnormal expression of actin in host tissues. Non-specific or polyclonal stimulation may be another mechanism to explain anti-actin antibody production (Bach *et al.*, 1986). However, we believe that paramyxoviruses can trigger the immune response to actin as a result of specific interaction between cellular actin and paramyxovirus M protein. The possibility that the interaction of actin and M protein contributes to the increase in the anti-actin antibody titers is intriguing. I postulate that this interaction leads to alteration of actin conformation rendering it antigenic to the host. Alternatively, M protein may act as a carrier in hapten-carrier phenomenon and provide help for anti-actin antibody production. Further study of this system as a possible model of a virus-induced autoimmune response seems warranted.

Mechanisms of virus-induced autoimmune responses.

The mechanism by which autoimmune responses arise remains one of the major problems in medicine. Autoimmune responses often accompany virus infection in man. Despite a great deal of speculation about the role of viruses in autoimmunity, there are relatively few examples where viruses can be directly incriminated as causative agents of an autoimmune diseases. However, with the advent of new

technologies, evidence is rapidly accumulating to support the existence of a possible etiological role for viruses in autoimmune diseases.

Although viruses have considerable potential to cause or at least trigger autoimmune phenomena, there are relatively few well-defined examples by which the mechanism of post-infectious autoimmunity can be explained. Viruses may mediate autoreactivity by either affecting the proliferative or effector phases of the immune response. The mechanisms by which virus infection can lead to the production of autoreactivity are grouped into three major categories: modified self, fortuitous immunological cross-reactivity, and changes in immune regulation (Rager-Ziman, 1988).

1. **Modified self**

1.1 Release of sequestered antigens.

Infection by cytolytic viruses can lead to cell destruction and tissue necrosis with the release of normal cell components. The once sequestered cellular components are recognized as foreign by the host immune system and treated in the same manner as any other foreign antigens.

1.2 Virus-induced alterations of host cell proteins.

During infection and replication in host cells, viruses may insert, expose or modify host antigens on the cell surface. Normally the immune system will not recognize the cell surface as foreign, but when viral antigens are expressed in association with membrane components, the immune system will recognize the complex as foreign. Studies of expression of cytoskeleton components in various Epstein-Barr virus-infected cell lines have shown that this virus can induce alteration in the content and distribution of the cytoskeleton structure, particularly actin microfilaments and microtubules (Zauli *et al.*, 1988). Furthermore, there is increased surface expression of actin in Epstein-Barr virus-transformed cells (Bachvaroff *et al.*, 1980). Autoantibodies to smooth muscle or actin have been found in sera of patients infected with this virus (Notkins *et al.*, 1984). Therefore, EBV may play a role by increasing the surface expression of actin which leads to the production of anti-actin antibodies in the host.

2. Fortuitous immunological cross-reactivity

2.1. Molecular mimicry.

This is a common phenomenon in which viral antigens share antigenic epitopes with normal host cell components. The homology between a viral protein and host cell protein is

the basis of antibody cross-reactivity. For example, monoclonal antibodies to the measles phosphoprotein cross-react with intermediate filaments of human cells (Fujinami *et al.*, 1983). A hybridoma obtained from vaccinia virus immunization produced antibodies with an affinity for the intermediate filament protein, vimentin, as well as a viral component (Dales *et al.*, 1982). Furthermore, molecular mimicry is implied by the fact that 4% of monoclonal antibodies raised against RNA and DNA viruses cross-react with uninfected host cell tissue (Oldstone, 1987; Srinivasappa *et al.*, 1986).

Computer searches for viral proteins bearing regions of homology with host proteins have been used successfully to identify some potential cross-reactivities (Fujinami and Oldstone, 1985). One of the similarities found was a sequence in myelin basic protein from amino acid 589 to 598 which was homologous to a sequence in hepatitis B polymerase. This particular peptide sequence of myelin basic protein is encephalitogenic for the rabbit. The relevant viral DNA polymerase synthetic peptide induces rabbit antibodies that cross-react with myelin basic protein. However, there has not been any evidence of an association of hepatitis B infection with any demyelinating diseases. Therefore, this homology appears to be only a fortuitous finding with no clinical significance.

2.2 Anti-idiotype antibodies.

Plotz (1983) has proposed that autoantibodies may be anti-idiotype antibodies to antiviral antibodies. According to this hypothesis, the anti-idiotype antibodies to virus neutralizing antibody may interact with the complementary virus receptor on the host cell, thereby acting as an autoantibody. That is to say, the anti-idiotype antibody and viral antigen will bind to the same site of the cell receptor. Mice immunized with measles virus developed antibodies which reacted with cell surface receptors for measles virus (Krah and Choppin, 1988). This observation supports the hypothesis that viruses may induce autoimmune responses against host cells through anti-idiotype antibodies to host cell membrane components.

3. Changes of immune regulation

3.1 Abnormal expression of immunoregulatory molecules.

Virus infections may also influence the immune system itself. They can significantly affect the expression of major histocompatibility complex (MHC) class I antigen (Brown *et al.*, 1988). This may be done indirectly through the release of soluble mediators, such as interleukins and interferons. Interferons can increase the expression of MHC antigens on the surface of neighboring cells. Alpha-interferon increases expression of MHC class I antigens which

are required for cytotoxic T-cell reactions, while gamma-interferon enhances the expression of MHC class II antigens on antigen-presenting cells which are required for T-helper cell interaction (Schattner, 1988).

Gamma-interferon was shown to induce HLA-DR (MHC class II antigen) on the surface of epithelial cells. These HLA-DR-positive cells could be recognized by T cell lines, derived from the thyroid of patients with Graves' disease, recognizing HLA-DR-positive thyrocytes (Feldman *et al.*, 1987). These observations suggest that somatic cells expressing class II antigens may play an active role in triggering an autoimmune response. If a viral infection can induce the expression of class II antigens on cells in infected tissue, this may be sufficient to elicit an autoimmune response (Feldman *et al.*, 1987).

3.2 Polyclonal B cell activation.

Autoantibodies directed against a wide range of normal tissue antigens have been found in the sera of animals and patients after certain viral infections. A panel of monoclonal autoantibodies prepared from reovirus type 1-infected mice contained multiple organ reactive autoantibodies (Haspel *et al.*, 1983). Multiple autoantibodies were also found in the sera of Balb/c, C57Bl/10 and C3H mice following infection with murine cytomegalovirus (MCMV) (Bartholomaeus *et al.*, 1988). Human

lymphoblastoid cell lines established after Epstein-Barr virus infection have been shown to produce a broad range of antibodies reacting with a panel of 13 self and non-self antigens such as trinitrobenzenesulfonic acid, the tripeptide L-glutamine-L-alanine-L-tyrosine (GAT), DNA, actin, tyroglobulin, and myoglobulin (Seigeneurin *et al.*, 1988).

3.3 Nonspecific adjuvant effects.

Adjuvants like bacterial cell walls and muramyl dipeptide are described as immune modulating agents. Their effects on the immune system include increased antigen processing, increased lymphocyte-macrophage interaction, increased helper cell activity, increased polyclonal B cell stimulation, increased production of interleukin-1, stimulation of macrophage protein synthesis and activation of macrophages (Sell, 1987). The mechanism of augmentation of the immune response to self by virus has been postulated to be in terms of an enhanced carrier-hapten effect. For example, NDV antigens act as helper determinants for the antibody responses to the cellular component, Thy-1, a T cell surface marker (Bromberg *et al.*, 1982). Autoimmune myocarditis in the A/J congenic mice is induced either by viral infection or immune response to cardiac myosin which is exposed to the immune system as a result of viral-mediated cell necrosis, and the inflammation associated with the immune response may exert an adjuvant effect (Rose *et al.*, 1988).

Rationale for this study

Actin has been identified as a structural component in several viruses including the paramyxoviruses. There is a considerable body of biochemical, morphological, and physiological evidence to support a role for actin microfilaments in the assembly process of some viruses, although this supposition remains to be proven. Clinical studies have shown that antibodies to actin are commonly found in the sera of patients after some viral infections. This is surprising since actin in its native form is a very weak antigen. It is unclear what initiates the immune response to actin after viral infection; however, the demonstration by Giuffre *et al.* (1982) that M protein interacts with actin suggested to me that the significance of the M protein-actin interaction warranted further study from the biochemical and immunological points of view. The fact that a specific viral protein was shown to interact with a specific host cell protein with subsequent production of autoantibodies to the host cell protein provided a possible model to study a mechanism for viral-induced autoimmunity. In this thesis, I have extended some of the original biochemical studies to further characterize the interaction of actin with M protein. In addition, I have examined the immune response to actin and the effect of different MHC genotypes on this response. I have also examined the effect of the M protein interaction with actin on the immune

response to actin. The results of these studies provide the first concrete evidence for a specific viral protein interaction with a defined host cell protein and the subsequent production of autoantibodies to the host cell protein.

In the final chapter, I have attempted to demonstrate the effect of M protein on the actin filaments in eukaryotic cells by the expression of M protein in fibroblasts. I felt that an *in vivo* demonstration of the M protein-actin interaction would lend more support for our hypothesis for a role of actin microfilaments in viral assembly and in an autoimmune response to actin.

In most of the studies I have used NDV M protein since the virus is easily grown and the M protein can be readily purified. It would have been much harder to produce sufficient measles virus and measles virus M protein for these studies. However, in the last chapter I have used the M protein gene from measles virus since we had this clone in our laboratory and we felt the principles of M protein interaction with actin microfilaments would be the same for all paramyxoviruses.

TABLE 1.1

Classification of *paramyxoviridae*.

Genus	Species	Host
Paramyxovirus	Newcastle disease virus (NDV)	bird
	Sendai virus (HVJ)	mouse
	Parainfluenza 1, 2, 3, 4	human
	Mumps virus	human
	Simian virus 5 (SV 5)	primates
Morbillivirus	Measles virus	human
	Canine distemper virus	dog
	Rinderpest	cattle
Pneumovirus	Pneumonia virus	mouse
	Respiratory syncytial virus (RS)	human bovine

MTEIYDFDKS AWDIKGSIAP IQPTTYSDGR LVPQVRVIDP GLGDRKDECF

MYMFLLGVVE DSDPLGPPIG RAFGSLPLGV GRSTAKPEKL LKEATELDIV

VRRTAGLNEK LVFYNNTPLT LLTPWRKVLT TGSVFNANQV CSAVNLIPLD

TPQRFRVVYM SITRLSDNGY YTVPRRMLEF RSVNAVAFNL LVTLRIDKAI

GPGKIIDNTE QLPEATFMVH IGNFRRKKSE VYSADYCKMK IEKMGLVFAL

GGIGGTSLHI RSTGKMSKTL HAQLGFKKTL CYPLMDINED LNRLLWRSRC

KIVRIQAVLQ PSVPQEFRIY DDVIINDDQG LFKVL

FIGURE 1.1 Predicted amino acid sequence of M protein of measles virus (Bellini *et al.*, 1986). Amino acid codes: A-alanine, C-cysteine, D-aspartic acid, E-glutamic acid, F-phenylalanine, G-glycine, H-histidin, I-isoleucine, K-lysine, L-leucine, M-methionine, N-asparagine, P-proline, Q-glutamine, R-arginine, S-serine, T-theonine, V-valine, W-tryptophan, Y-tyrosine.

Sarcomeric α:	Ac-DEDETT	ALVCDN	GSGLVK
Cardiac muscle α:	Ac-DDEETT	ALVCDN	GSGLVK
Smooth muscle α:	Ac-EEEDST	ALVCDN	GSGLCK
Smooth muscle γ:	Ac-DDDTT	ALVCDN	GSGLCK
Nonmuscle β:	Ac-DDDIA	ALVVDN	GSGMCK
Nonmuscle γ:	Ac-DDDIA	ALVIDN	GSGMCK

Figure 1.2 Amino-terminal sequence of actin isoforms (Bulinski *et al.*, 1983). Amino acid codes are the same as in Fig. 1.1. Ac is an abbreviation for acetylated N-terminal peptide.

BIBLIOGRAPHY

Andersen, P. and Andersen, H.K. 1975. Smooth-muscle antibodies and other tissue antibodies in cytomegalovirus infection. Clin.Exp.Immunol. 22: 22-29.

Andjaparidze, O.G., Chaplygina, N.M., Bogomolova, N.N., Koptyaeva, I.B., Nevryaeva, E.G., Filimonova, R.G., and Tareeva, T.E.. 1989. Detection of measles virus genome in blood leucocytes of patients with certain autoimmune diseases. Arch. Virol. 105: 287-291.

Bach, M.A., Lewis, D.E., McClure, J.E., Parikh, N., Rosenblatt, H.M., and Shearer, W.T. 1986. Monoclonal anti-actin antibody recognizes a surface molecule on normal and transformed human B lymphocytes: expression varies with phase of cell cycle. Cellular immunology. 98: 364-374.

Bachvaroff, R.J., Miller, F., and Rapaport, F.T. 1980. Appearance of cytoskeletal components on the surface of leukemia cells and of lymphocytes transformed by mitogens and Epstein-Barr virus. Proc. Natl. Acad. Sci. U.S.A. 77: 4979-4983.

Baczko,K., Carter, M.J., Billeter, M., and ter Meulen, V. 1984. Measles virus gene expression in subacute sclerosing panencephalitis. Virus Research. 1: 585-595.

Baltimore, D. 1971. Expression of animal viral genomes. Bacteriol. Rev. 35: 235-241.

Barden, J.A., dos Remedios, C.G. 1983. High resolution proton NMR studies on G-actin. In: actin: structure and function in muscle and non-muscle cells. dos Remedios, C.G. and Barden, J.A., (eds.), pp. 53-62. Academic Press, New York.

Bartholomaeus, W.N., O'Donoghue, H., Foti, D., Lawson, C.M., Shellam, G.R., and Reed, W.D. 1988. Multiple autoantibodies following cytomegalovirus infection: virus distribution and specificity of autoantibodies. Immunology. 64: 397-405.

Bellini, W., Englund, G., Rozenblatt, S., Arnheiter, S., and Richardson, C. 1985. Measles virus P gene codes for two proteins. J. Virol. 53: 908-919.

Bellini, W.J., Englund, G., Richardson, C.D., Rozenblatt, S., and Lazzarini, R.A. 1986. Matrix genes of measles virus and canine distemper virus: cloning, nucleotide sequences and deduced amino acid sequences. J Virol. 58: 408-416.

Benyamin, Y., Roustan, C., and Boyer, M. 1986. Anti-actin antibodies: chemical modification allows the selective production of antibodies to the N-terminal region. J. Immunol. Methods. 86: 21-29.

Bias, W.B., Reveille, J.D., Beaty, T.H., Meyers, D.A., and Arnett, F.C. 1986. Evidence that autoimmunity in man is a mandelion dominant trait. Am. J. Hum. Genet. 39 (5): 584-602.

Black, F.L. 1988. Persistent measles virus genome in autoimmune chronic active hepatitis: cause or coincidence? Hepatology. 8 (1): 186-187.

Blumberg, B.M., Rose, K., Simona, M.G., Rouse, L., Giorgi, C., and Kolakofsly, D. 1984. Analysis of the Sendai virus M gene and protein. J.Virol. 52: 656-663.

Bohn, W., Rutter, G., Hohenberg, H., and Mannweiler, K. 1983. Inhibition of measles virus budding by phenothiazines. Virology. 130 (1): 44-55.

Bohn, W., Rutter, G., Hohenberg, H., Mannweiler, K., and Nobis, P. 1986. Involvement of actin filaments in budding of measles virus: studies on cytoskeletons of infected cells. Virology. 149 (1): 91-106.

Bromberg, J.S., Lake, P., and Brunswick, M. 1982. Viral antigens act as helper determinants for antibody response to cell surface antigens. J. Immunol. 129: 683-688.

Brown, G.D., Choi, Y., Pampeno, C., and Meruelo, D. 1988. Regulation of H-2 class I gene expression in virally transformed and infected cells. CRC critical reviews in immunology. 8 (3): 175-215.

Bulinski, J.C., Kumar, S., Titani, K., and Hauschka, S.D. 1983. Peptide antibody specific for the amino terminus of skeletal muscle α-actin. Proc. Natl. Acad. Sci. U.S.A. 80: 1506-1510.

Cattaneo, R., Schmid, A., Rebmann, G., Baczko, K., ter Meulen, V., Bellini, W.J., Rozenblatt, S., and Billeter, M. 1986. Accumulated measles virus mutations in a case of subacute sclerosing panencephalitis: interrupted matrix protein reading frame and transcription alteration. Virology. 154: 97-107.

Chambers, P., Millar, N.S., Platt, S.G., and Emmerson, P.T. 1986. Nucleotide sequence of the gene encoding the

matrix protein of Newcastle disease virus. Nucleic Research. 14 (22): 9051-9061.

Chapel, H., and Haeney, M. 1984. Joints and muscles. In: Essentials of clinical immunology. pp. 188-219. Blackwell Scientific Publications, Oxford, London.

Choppin, P. and Compans, R. 1975. Reproduction of paramyxoviruses. In: Comprehensive virology, Vol. 4 (Fraenkel-Conrat, H. and Wagner, R.R. eds.), pp. 95-178. Plenum Publishing Corp., New York.

Christie, K.E., Endresen, C., and Haukenes, G. 1984. IgM antibodies in sera from patients with chronic active hepatitis reacting with the measles virus matrix protein and nucleoprotein, J. Med. Virol. 14: 149-157.

Christie, K.E.and Haukenes, G. 1982. Characterization of measles virus-specific antibodies in sera from patients with chronic active hepatitis. Journal of Hygiene. 88: 519-529.

Closs, O., Haukenes, G., Gjone, E., and Blomloff, J.P. 1971. Raised antibody titers in chronic liver disease. Lancet. 2: 1202-1203.

Cooper, J.A. 1987. Effects of cytochalasin and phalloidin on actin. J. Cell. Biol. 105: 1473-1478.

Cruse, J.M. and Lewis,Jr., R.E. 1988. The genetic basis of aberrant autoimmune-regulation. Concepts. Immunopathol. 5: 1-20.

Dales, S., Fujinami, R.S., and Oldstone, M.B.A. 1983. Infection with vaccinia favors the selection of hybridomas synthesizing autoantibodies against intermediated filaments, one of them cross-reacting with the virus heamagglutin. J. Immunol. 131: 1546-1553.

Damsky, C., Sheffield, J., Tuszynski, G., and Warren, L. 1977. Is there a role for actin in virus budding? J. Cell. Biol. 75: 593-605.

Doniach, D., Bottazzo, G.F., and Cudworth, A.G. 1983. Etiology of type I diabetes mellitus: heterogeneity and immunological events leading to clinical onset. Ann. Rev. Med. 34: 13-20.

Dubois-Dalcq, M., Holmes, K.V., Rentier, B., and Kingsbury, D.W. 1984. Assembly of enveloped RNA viruses, pp. 44-65. Springer-Verlag, Wein , New York.

Elango, N. 1989. Complete nucleotide sequence of the matrix protein mRNA of mumps virus. Virology. 168: 426-428.

Enami, M., Sato, T.A., and Sugiura, A. 1989. Matrix protein of cell-associated subacute sclerosing panencephalitis viruses. J. Gen. Virol. 70: 2191-2196.

Evans, A.E.and Comstocks, G.W. 1981. Presence of elevated antibody titer to EBV before Hodgkins disease. Lancet. 1: 1183-1186.

Faaberg, K.S., and Peeples, M.E. 1988. Strain variation and nuclear association of Newcastle disease virus matrix protein. J. Virol. 62: 586-593.

Fagraeus, A., and Norberg, R. 1978. Anti-actin antibodies. Curr. Top. Microbiol. Immunol. 82: 1-13.

Fagraeus, A., Orvell, C., Norberg, R., and Norrby, E. 1983. Monoclonal antibodies to epitopes shared by actin and vimentin obtained by paramyxovirus immunization. Exp. Cell. Res. 145: 425-432.

Fagraeus, A., Tyrrell, D.L.J., Norberg, R., and Norrby, E. 1978. Actin filaments in paramyxovirus-infected human fibroblasts studied by indirect immunofluorescence. Arch. Virol. 57: 291-296.

Farrow, L.J., Holborow E.J., Johnson , G.D., Lamb, S.G., Stewart,J.S., Taylor, P.E., and Zuckerman, A.J. 1970. Autoantibodies and hepatitis-associated antigen in acute infective hepatitis. Br. Med. J. 2: 693-695.

Feldman, M. 1987. Regulation of HLA class II expression and its role in autoimmune diseases. In Autoimmunity and autoimmune disease. Ciba Foundation Symposium 129. Evered, D. and J. Whelan, (eds.), pp. 88-108. John Wiley &Sons U.K.

Fujinami, R.S., and Oldstone, M.B.A. 1985. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. Science. 230: 1043-1045.

Fujinami, R.S., Oldstone, M.B.A., Wroblewska, Z., Frankel, M.E., and Koprowski, H. 1983. Molecular mimicry in virus infection; cross reaction of measles phosphoprotein or of herpes simplex virus protein with human intermediated filaments. Proc. Natl. Acad. Sci. U.S.A. 80: 2346-2350.

Gabbiani, G., Graeme, B.R., Lamelin, J.P., Vassalli, P., Majno, G., Bouvier, C.A., Cruchaud, A. and Luscher, E.F. 1973. Human smooth muscle antibodies: its identification as an anti-actin antibody and a study of binding to nonmuscular cell. Am. J. Pathol. 72: 473-488.

Garrel, J.I., and Gibson , W. 1976. Identification and characterization of multiple forms of actin. Cell. 9: 793-805.

Giorgi, C., Blumberg, B., and Kolakofsky, D. 1983. Sendai virus contains overlapping genes expressed from a single mRNA. Cell. 35: 829-836.

Giuffre, R.M., Tovell, D.R., Kay, C.M., and Tyrrell, D.L.J. 1982. Evidence for an interaction between membrane protein of a paramyxovirus and actin. J. Virol. 42: 963-968.

Griffin, J.A., and Compans, R.W. 1979. Effect of cytochalasin B on the maturation of enveloped viruses. J. Exp. Med. 150: 379-391.

Gruber, C. and Levine, S. 1983. Respiratory syncytial virus III: the envelope associated proteins. J Gen Virol. 64: 825-832.

Haire, M. 1972. Fibrillar anti-cellular antibody associated with mumps and measles infection. Clin. Exp. Immunol. 12: 335-341.

Holborow, E.J., Hemsted, E.H. and Meads, S.V. 1973. Smooth muscle autoantibodies in infectious mononucleosis. Br. Med. J. 3: 323-325.

Hall, W.W., Lamb. R.A., and Choppin, P.W. 1979. Measles and subacute sclerosing panencephalitis virus proteins: lack of antibodies to the M protein in patients with subacute sclerosing panencephalitis. Proc. Natl. Acad. Sci. U.S.A. 76: 2047-2051.

Hambly, B.D. Raison, R.L., and dos Remedios, C.G. 1983. Monoclonal antibodies directed against skeletal muscle actin. Biochemistry International 7(6): 739-746.

Haspel, M.V., Onodera, T., Prabhakar, B.S., McClintock, P.R., Essani, K., Ray, U.R., Yagihashi, S., and Notkins, A.L. 1983. Multiple organ-reactive monoclonal autoantibodies. Nature 303: 73-76.

Heggeness, M. Smith, P. and Choppin, P. 1982. *In vitro* assembly of the nonglycosylated membrane protein (M) of Sendai virus. Proc. Natl. Acad. Sci. U.S.A. 75: 6232-6236.

Henson, T.E., Brody, J.A., Sever, J.L.. 1970. Measles antibody titers in multiple sclerosis patients, siblings and controls JAMA. 211: 1985-1988.

Hightower, R.C., and Meagher, R.B. 1986. The molecular evolution of actin. Genetics. 114(1): 315-332.

Hiruma, S., and Hashimoto, S. 1988. Monoclonal antibody to rat brain actin antigenically enhanced with HVJ (Sendai virus) M protein. Histochemistry 90: 1-8.

Hue, H.K., Labbe, J.P., Harricane, M.C., Cavadore, J.C., Benyamin, Y., and Roustan, C. 1988. Structural and functional variations in skeletal-muscle and scallop muscle actins. Biochem. J. 256: 853-859.

Jockusch, B.M., Kelly, K.H., Meyer, R.K., and Burger, M.M. 1978. An efficient method to produce specific anti-actin. Histochemistry 55: 177-184.

Johnson, G.O., Halborow, E.J., and Glynn, L.E. 1965. Antibody to smooth muscle in patients with liver disease. Lancet 2: 878-890.

Johnson, R.T. 1987. The pathogenesis of acute viral encephalitis and post-infectious encephalomyelitis. J. Infectious Diseases. 155(3): 359-364.

Kalland, K.H., Endresen, C., Haukenes, G., and Schrumpf, E. 1989. Measles-specific nucleotide sequences and autoimmune chronic active hepatitis. Lancet. 1: 1390-1391.

Krah, D.L. and Choppin, P.W. 1988. Mice immunized with measles virus develop antibodies to a cell surface receptor for binding virus. J. Virol. 62(5): 1565-1572.

Kyte, J., and Doolittle, R.F. 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157: 105-132.

Lamb, R.A. and Choppin, P. W. 1977. The synthesis of Sendai virus polypeptide in infected cells. III Phosphorylation of polypeptides. Virology 81: 382-397.

Lamb, R., Mahy, B., and Choppin, P. 1976. The synthesis of Sendai virus polypeptides in infected cells. Virology 69: 116-131.

Lazarides, E., and Weber, K. 1974. Actin antibodies: The specific visualization of actin filaments in non-muscle cells. Proc. Natl. Acad. Sci. U.S.A. 71: 2268-2272.

Lucas, C.J., Galama, J.M.D., and Ubels-Postma, J.C. 1979. Lymphocyte and measles virus. In: virus-lymphocyte interactions: implications for disease.Proffit, M.R.,

(ed.), pp. 243-250. Elsevier-North-Holland, Inc. New York.

Lund, G.A., Tyrrell, D.L.J., Bradley, R.D., and Scraba, D.G. 1984. The molecular length of measles virus DNA and the structural organization of measles nucleocapsids. J. Gen. Virol. 65: 1535-1542.

Mathews, R. 1982. Classification and nomenclature of viruses. Intervirology. 17:1-181.

McGlure, J.E., and Shearer, W.T. 1988. Radioimmunoassay for anti-actin antibody: application in viral and autoimmune diseases. Mol. Cell. Probes. 2(4): 305-319.

Meyer, R.K., Burger, M.M. Tschannen, R., and Schafer, R. 1981. Actin filament bundles in vaccinia virus infected fibroblasts. Arch. Virol. 67: 11-18.

Moench, T.R., Griffin, D.E., Obriecht, C.R., Vaisberg, A.J., and Johnson, R.T. 1988. Acute measles in patient with and without neurological involvement: distribution of measles virus antigen and RNA. J. Infect. Dis. 158: 433-442.

Morrison, T.G. 1988. Structure, function, and intracellular processing of paramyxovirus membrane proteins. Virus Research 10: 113-136.

Morrison, T.G. and McGinnes, L.J. 1985. Cytochalasin D accelerates the release of Newcastle disease virus from infected cells. Virus Research 4(1): 93-106.

Nishioka, M., Aibiki, T., Shirai, M., Terada, S., Hagawa, H., and Watanabe, S. 1986. Rabbit autoantibodies to actin induced by immunization with modified homologous actins. Microbiol. Immunol. 30(12): 1291-1297.

Norrby, E. 1985. Measles virus. In: "Virology" Fields, B.N., (ed.), pp 1305-1321. Raven Press, New York.

Notkins, A.L., Onodera, T. and Prabhakar, B. 1984. Virus-induced autoimmunity. In: Concepts in viral pathogenesis, Notkins, A.L. & Oldstone, M.B.A. (eds.), pp. 210-215. Vienna & New York, Springer-Verlag.

Oldstone, M.B.A., 1987. Molecular mimicry and autoimmune disease. Cell 50: 819-820.

Peeples, M.E. 1988. Differential detergent treatment allows immunofluorescent localization of the Newcastle disease virus matrix protein within the nucleus of infected cells. Virology 162: 255-259.

Plotz, P.H., 1983. Autoantibodies are anti-idiotype antibodies to anti-viral antibodies. Lancet 2: 824-826.

Portocala, R., Spyrou, N., Lambropoulou, V., and Pateraki, E. 1988. The presence of both antivirus and antiself antibodies in sera from patients with adenovirus and influenza B. Virologie.39(3): 207-216.

Rager-Zisman, B. 1988. Virus-induced autoimmunity: Israel. J. Med. Sci. 24(7): 363-365.

Richardson, C.D., Berkovich, A.,Rozenblatt, S., and Bellini, W.J. 1985. Use of antibodies directed against synthetic peptides for identifying cDNA clones, establishing reading frames, and deducing the gene order of measles measles virus. J. Virol. 54: 186-193.

Robertson, D.A.F., Zhang, S.L., Guy, E.C., and Wright, R. 1987. Persistent measles virus genome in autoimmune chronic active hepatitis. Lancet 2: 9-11.

Rose, N.R., Herskowitz, A., Neumann, D.A., and Neu, N. 1988. Autoimmune myocarditis. Immunology Today 9(4): 117-120.

Roustan, C. Benyamin, Y., Boyer, M., and Cavadore, J.C. 1986. Structural variations in actins: a study of the immunological reactivity of the N-terminal region. Biochem. J. 233: 193-197.

Roustan, C. Benyamin, Y., Boyer, M., Bertrand, R., Audemark,E., and Jauregui-Adell J. 1985. Conformational changes induced by Mg^{2+} on actin monomers: an immunologic attempt to localize the affected region. FEBS Letters. 181(1): 119-123.

Schattner, A. 1988. Lymphokines in autoimmunity - roles of interferons in systemic lupus erythematosus and other autoimmune disorders. Israel J. Med. Sci. 24(12): 728-731.

Scheid, A. and Choppin, P.W. 1977. Two disulfide-linked polypeptide chains constitute the active F protein of paramyxoviruses. Virology 80:54-66.

Seigeneurin, J.M. Guilbert, B. Bourgeat, M.J., and Avrameas, S. 1988. Polyspecific natural antibodies and autoantibodies secreted by human lymphocytes immortalized with Epstein-Barr virus. Blood 71(3): 581-585.

Sell, S. 1987. Immunolgy, Immunopathology and immunity. 4th. edition. pp.669. Elsevier Science Publishing Company, Inc. New York.

Shoenfeld, Y. and Isenberg, D.A. 1989. The mosaic of autoimmunity. Immunology Today 10(4): 123-126.

Srinivasappa, J., Saegusa, J., Prabhakar, B.S., Gentry, M.K., Buchmeier, M.J., Wikir, T.J., Koprowski, H., Oldstone, M.B.A., and Notkins, A.L. 1986. Molecular mimicry: frequency of reactivity of monoclonal antiviral antibodies with normal tissues. J. Virol. 57: 397-401.

Stallcup, K.C., Raine, C.S., Fields, B.N. 1983. Cytochalasin B inhibits the maturation of measles virus . Virology 124(1); 59-74.

ter Meulen, V., Stephenson, J.R., and Kreth, H.W. 1983. Subacute sclerosing panencephalitis. In "Comprehensive Virology", Fraenkel-Conrat, H. and Wagner, R.R. (eds.), Vol 18, pp. 105-142. Plenum, New York.

Trenchev, P., and Holborow, E.J. 1976. The specificity of anti-actin serum. Immunology 31: 509-517.

Tuffereau, C., and Roux., L. 1988. Direct adverse effects of Sendai virus DI particles on virus budding and on M protein fate and stability. Virology 162: 417-426.

Tyrrell, D.L.J. and Norrby, E. 1978. Structural polypeptides of measles virus. J. Gen. Virol. 39: 219-229.

Tyrrell, D.L.J., and Ehrnst, A. 1979. Transmembrane communication in cells chronically infected with measles virus. J. Cell Biology 81: 396-402.

Van Regenmortel, M.H.C. 1986. Which structural features determine protein antigenicity? Trends Biochem. Sci. 11: 36-39.

Vandekerckhove J., and Weber, K. 1984. Chordate muscle actins differ distinctly from invertebrate muscle actins: The evolution of the different vertebrate muscle actin. J. Mol. Biol. 179: 391-413.

Vandekerckhove, J., and Weber, K. 1978,. At least six different actins are expressed in a higher mammal: an analysis based on the amino acid sequence of the amino-terminal tryptic peptide. J. Mol. Biol. 126: 783-802.

Vandekerckhove, J., DeCouet, H.G. and Weber, K. 1983. Molecular evolution of muscle-specific actins: a protein chemical analysis. In: Actin, Structure and Function in Muscle and Non-Muscle Cells., dos Remedios C. and Barden J.A., (eds.), pp. 241-248, Academic Press, New York.

Wang, E., Wolf, B.A. Lamb, R.A., Choppin, P.W., and Goldberg, A.R. 1976. The presence of actin in enveloped virus. In: Cell motility. Goldman, R., Pollard, T., and Rosenbum, J. (eds.), pp.589-600. Cold Spring Harbor Press, Cold Spring Harbor, New York.

Wong, T.C., Wipe, G. and Hirano, A. 1987. The measles virus matrix gene and gene product defined by *in vitro* and *in vivo* expression. Virology. 157: 497-508.

Yoshida, T., Nagai, Y., Yoshi, S., Maneo, K., Mutsumoto, T., and Hashino, M. 1976. Membrane (M) protein of HVJ (Sendai virus): its role in virus assembly. Virology. 71: 143-161.

Zauli, D., Musiani, M., Crepsi, C., and Zerbini, M. 1988. Expression of cytoskeleton components in various Epstein-Barr virus infected cell lines. Pathology. 20: 105-108.

CHAPTER 2

Interaction of Actin and Paramyxovirus M Protein[1,2]

Actin

Actin is a major component of muscle thin filaments in the cytoskeleton. It is highly conserved during evolution. The actin molecule is composed of a single polypeptide of 375 amino acids. In low salt solutions, it is known as a globular actin or G actin, but in high salt solutions, actin can polymerize to form actin filaments or the so-called filamentous or F actin. Approximately 50% of the actin molecules in most eukaryotic cells exist in unpolymerized form either as free monomers or as complexes with other proteins (Albert *et al.*, 1989). The dynamic equilibrium of the actin pool between G actin and F actin helps drive the surface movements of cells. An actin binding protein, filamin, crosslinks the actin filaments together into a three-dimensional network of actin whereas gelsolin breaks down the crosslinked network of actin filaments in the presence of Ca^{+2}.

---

[1] A version of this chapter has been published: (1) Anomasiri, W.T.,Tovell, D.R., and Tyrrell, D.L.J. 1990. J. Virol. (in press). (2) Anomasiri, W.T., Armstrong, G.D., and Tyrrell, D.L.J.1990. J. Virol.(Submitted).

[2] The former publications are written under "Anomasiri", my married name.

Actin-binding proteins

More than fifty proteins have been found to bind to actin (Stossel *et al.*, 1985). Some bind to actin monomers, whereas others bind to actin filaments either at the ends of the filaments or along their sides. The crosslinking proteins can crosslink actin filaments to each other or to other structures such as the plasma membrane. The characteristics of some actin monomer-sequestering proteins are shown in Table 2.1.

The actin monomer-sequestering proteins bind to actin monomers but they do not form stable complexes with actin filaments. Upon binding to the ends of the filaments, these proteins will prevent the elongation of the actin filaments until the sequestering protein-actin complex dissociates from the filament ends. For example, DNaseI and profilin bind to both monomeric actin and filament ends and retard the polymerization of actin. Other actin monomer-sequestering proteins, such as depectin, may bind to actin subunits in the interior of filaments, causing filament breakage as well as sequestration of monomers.

Pancreatic deoxyribonuclease I (DNaseI) acts as both an actin monomer-sequestering protein and a filament-severing protein. It forms a tight 1:1 complex with actin which is strong enough to depolymerize F actin (Lazarides and Lindberg, 1974) and inhibit the enzymatic activity of DNaseI

(Lacks, 1981). The binding constants for DNaseI binding to monomeric actin and to filamentous actin are $5x10^8$ M^{-1} and $1.2x10^4$ M^{-1} respectively (Mannherz *et al.*, 1980). The two contact areas for the binding of actin to DNaseI are located in the segments 48-92 (Sutoh, 1984) and 168-226 (Boyer *et al.*, 1985) of actin. Vitamin D-binding protein (Gc globulin), which is a mammalian serum protein, binds to actin in a manner similar to profilin. Upon binding to actin, it can depolymerize actin filaments. The binding sites of vitamin D-binding protein and DNaseI are different since the complex of actin and vitamin D-binding protein can bind to DNaseI with the same binding constant as actin alone binds to DNaseI (Goldschmidt-Clermont *et al.*, 1985).

In addition to some cellular proteins binding to actin, paramyxovirus M protein has been shown to bind to actin *in vitro* (Giuffre *et al.*, 1982). M protein forms a shell on the inner surface of the viral envelope. It can self-assemble into an ordered structure or associate with other viral proteins during viral budding (Yoshida *et al.*, 1976). It is also thought to interact with cellular actin during the assembly of progeny virions in paramyxovirus-infected cells (Tyrrell and Ehrnst, 1979; Bohn *et al.*, 1986). Newcastle disease virus (NDV) M protein specifically interacts with rabbit muscle actin as shown by affinity chromatography, circular dichroism and cosedimentation (Giuffre *et al.*, 1982). In this chapter I have extended these original

observations by determining the binding constant of M protein and actin. These have been compared to the binding constant of actin and DNaseI. I have also done some of the mapping of the binding sites for M protein on actin using affinity chromatography and modified ELISA assays. In addition, the binding of M protein to carbonic anhydrase, another skeletal muscle protein (Vaananen *et al.*, 1988), is described in this chapter.

MATERIALS AND METHODS

Preparation of actin and tryptic-fragment of actin. Actin was purified from acetone powder prepared from rabbit skeletal muscle by the method of Spudich and Watt (1971). The purity of the actin was verified on SDS-PAGE. Purified actin was digested with trypsin as described by Jacobson and Rosenbusch (1976). Tryptic cleavage of actin yielded a major fragment comprising residues 63-375 (core) and small peptides. The major fragment of the trypsin digest was identified by comparison with Coomassie blue stained gels. The protein bands were cut from preparative SDS-PAGE gels, and the proteins were electroeluted for use in the affinity chromatography and ELISA assays.

Preparation of M protein from Newcastle disease virus (NDV). Nine-day-old embryonated eggs were infected with 0.1-0.2 ml of the stock NDV injected into the allantoic cavity using a 23-gauge needle. The holes in the eggs were sealed with hot wax and the eggs were incubated at 37 °C with the air sac uppermost. Four days after incubation, the eggs were chilled at 4 °C for at least 2 h and the allantoic fluids were harvested by removing the egg shell over the air sac followed by disruption of the chorioallantoic membrane and aspirating the allantoic fluid with a sterile 10 ml pipet.

The allantoic fluids were clarified by centrifugation at 4,000 rpm for 10 min at 4 °C in order to remove membrane and cellular debris. The supernatants were centrifuged at 10,000 rpm for 1 h to sediment the virus particles. The pellets were resuspended in PBS and sonicated for about 10-15 seconds. The virus suspensions were centrifuged through step gradients of 15% and 30% sucrose for 3 h at 25,000 rpm. The viral band at the interface was collected as purified virus stock and used for M protein preparation.

The M protein of Newcastle disease virus (NDV) was purified using the method previously described by Scheid and Choppin (1973). The virus was disrupted with 2% Triton X-100 in 1 M KCl in 0.01 M sodium phosphate (pH 7.3) and insoluble proteins were removed by sequential centrifugation at 10,000 x g for 30 min and 230,000 x g for 1 h. The M protein was precipitated from the supernatant by dialysis against a low salt buffer (0.01 M sodium phosphate, pH 7.3). The M protein was pelleted by centrifugation at 10,000 xg for 30 min and resuspended in 1 M KCl in 0.01 M sodium phosphate buffer, pH 7.3.

Myoglobin, bovine serum albumin (BSA), bovine DNaseI and carbonic anhydrase were purchased from Sigma and their purity was confirmed by SDS-PAGE and staining with Coomassie blue. They were used without further purification. These protein solutions were prepared in PBS.

Affinity chromatography was performed as previously described by Giuffre *et al.* (1982). NDV M protein (0.5 mg) was covalently linked to 2 g of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Inc.). Three-ml columns were prepared with these beads equilibrated in 0.14 M NaCl, 0.02 M sodium phosphate, pH 7.4 (PBS) and washed with cytochrome C (10 mg in 1 ml of PBS) to prevent non-specific adsorption of labelled proteins. A mixture of the three labelled proteins (actin, BSA and myoglobin) (10 µg and 1 x 10^6 cpm of each ^{125}I-protein) in PBS was passed through the column. The proteins were eluted with 2 M KCl and 8 M urea and identified by SDS-PAGE followed by autoradiography.

Protein iodination. Actin, bovine serum albumin (BSA) and myoglobin were radiolabelled with ^{125}I using iodo-beads (Pierce Chemical Company). Three hundred micrograms of each protein were incubated with an iodo-bead and 1 µl of Na^{125}I (17 Ci/mmole) in PBS at 37 °C for 1 h. The solution was removed from the bead and loaded on a Sephadex G-25 column prewashed with 1% BSA and PBS in order to separate the radiolabelled protein from the radioactive iodide. Iodinated proteins were purified on a Sephadex G-25 column and the specific activity of the labelled proteins was determined as described by Armstrong and Keppler (1987).

Protein binding assay. Equilibrium binding assays were performed at room temperature in 12 x 75 mm polystyrene

tubes essentially as described by Tyrrell *et al.* (1989). Each tube was coated with 100 µl of 5 µg/ml unlabelled protein at room temperature overnight. The tubes were then washed three times with 0.5 ml PBS containing 0.1% Tween 20 (PBS-Tween). The non-specific protein binding sites were then saturated with 0.5% BSA in PBS-Tween for 4 hours at room temperature. Next, the tubes were washed three times with PBS-Tween and incubated for 1 h with radiolabelled ligand (3.6 nM to 100 nM), with or without an excess of unlabelled ligand (10 µM). The total assay volume was 0.16 ml. After incubation, the tubes were washed four times with 0.5 ml of 0.1% BSA in PBS-Tween and the radioactivity was measured in a gamma counter (LKB RACK Gamma 5500). All assays were performed in triplicate and the equilibrium binding data were analyzed by the Scatchard procedure (Berzofsky *et al.*, 1989).

Competitive binding assay. The competitive binding assay was performed in a manner similar to the equilibrium binding assays with the exception that virus M protein-coated polystyrene tubes were incubated with 0.02 µM ^{125}I-actin and various concentrations (0.1 µM to 10 µM) of unlabelled actin, M protein, unlabelled but iodinated M protein, DNaseI or carbonic anhydrase.

Preparation of rabbit antisera. Actin (1.0 mg) was mixed with M protein (0.5 mg) and G-actin buffer was added to a total volume of 1 ml. The solution was left for 30 min at

22 °C prior to emulsification in complete Freund's adjuvant (CFA). Dutch rabbits were immunized by intramuscular injection and received booster injections of an equivalent amount of the proteins in incomplete Freund's adjuvant (IFA) at 4 and 5 weeks. Sera were taken at 6 weeks post immunization and tested for antibody production by ELISA.

Actin (1.0 mg) was mixed with 1 ml of M protein buffer. This was used in a similar immunization schedule to produce anti-actin antibodies. M protein (0.5 mg) was mixed with an equivalent amount of G-actin buffer and used to immunize Dutch rabbits in the same schedule as described above to produce anti-M protein antibodies.

Enzyme linked immunosorbent assay (ELISA)

Micro ELISA plates were coated with various amounts of actin, tryptic fragment of actin, BSA, or myoglobin at 0 ng to 200 ng per well in PBS. After overnight incubation at 4 °C, the plates were washed with PBS containing 0.1% Tween 20 (PBS-Tween). The plates were incubated with 100 µl (1 mg/ml) of NDV M protein for 1 h at 37 °C followed by washing five times with PBS-Tween. Rabbit sera were diluted 1:2000, and 100 µl was added to each well and incubated at 37 °C for 1 h. After washing the plates four times with PBS-Tween, 100 µl of peroxidase conjugated with goat anti-rabbit immunoglobulin (Cappel Laboratories) was added and the plates were incubated at 37 °C for 1 h. The plates were washed 5 times with PBS-Tween, and 100 µl of H_2O_2 and 2,2'-azino, di [3-ethyl-

benzthiazoline sulfonate] (ABTS) was added. Plates were incubated at 37 °C for 20 min and absorbance was read at 414 nm in a Titertrek multiscan photometer.

RESULTS

Analysis of interaction by affinity chromatography. Affinity columns were prepared with NDV M protein bound to Sepharose 4B. When a mixture of ^{125}I-labelled proteins (actin, BSA and myoglobin) was passed through a control column (CNBr-Sepharose 4B), there was no evidence that any of the protein bound to the beads (Fig. 2.1, panel A). However, when the mixture of ^{125}I-labelled proteins was passed through the affinity column, actin was selectively bound by the M protein-Sepharose 4B beads and could be partially eluted with 2 M KCl and 8 M urea (Fig. 2.1, panel A). These data confirm the results of Giuffre *et al.* (1982). In this study, I have extended these observations to include the binding of the major tryptic fragment of actin (33 kDa) as shown in Fig. 2.1, panel B. The major tryptic fragment of actin was also bound to M protein, but less tightly than the undigested actin.

Demonstration of the interaction of M protein with actin by ELISA. Additional evidence for the interaction of M protein with actin or the tryptic fragment of actin (33 kDa) was obtained by ELISA, as shown in Fig. 2.2. Micro-ELISA plates were coated with actin, the tryptic fragment of actin, BSA or myoglobin at 0-200 ng protein in 100 µl of PBS per well. After incubation overnight at 4 °C, the plates were washed 4x with PBS-Tween and incubated with

100 µl of 1 mg/ml of M protein for 1 h at 37 °C. The plates were washed as above and incubated with rabbit anti-M protein antisera for 1 h at 37 °C. The plates were washed again and incubated with peroxidase-labelled goat anti-rabbit immunoglobulin. After 1 h, the plates were washed 5 times, the ELISA substrate was added and the absorbancy at 414 nm read after 20 min. The results confirmed that M protein bound specifically to actin but not to BSA or myoglobin (Fig.2.2). M protein also bound to the tryptic fragment of actin but less well than it bound to native actin. This could suggest that the binding site of M protein on actin is located near amino acid residue 63, since digestion of actin with trypsin decreases but does not totally abolish binding. Alternatively, removal of the 63 amino acids could have an effect on the conformation of the trypsin-resistant fragment which alters M protein binding at a site removed from the cleavage site.

Measurement of dissociation constant. Equilibrium binding experiments were performed to characterize the interaction of ^{125}I-labelled actin and paramyxovirus M protein coated on polystyrene tubes. Scatchard analysis of the data obtained from these experiments revealed that the dissociation constant (K_D) for the ^{125}I-labelled actin-NDV M protein complex was 6.3×10^{-8} M (Fig. 2.3). In contrast, when ^{125}I-labelled M protein was used in binding experiments with actin-coated polystyrene tubes, the reaction did not reach

saturation in the concentration range used. The data obtained from the competitive binding experiments indicate that iodinated-M protein binds less well to actin than unlabelled M protein (Table 2.2). This observation implies that M protein contains one or more tyrosine residues which are important for its actin-binding activity or iodination alters the "native" conformation of at least a portion of M protein.

The interaction of virus M protein and bovine carbonic anhydrase was also studied using ^{125}I-labelled M protein. The specific binding of ^{125}I-M protein to carbonic anhydrase reached a plateau and the K_D obtained from Scatchard analysis of the data was 1.0×10^{-7} M (Fig.2.4). Therefore, it was apparent that tyrosine groups were not important for M protein binding to carbonic anhydrase. Carbonic anhydrase did not block the ability of ^{125}I-actin to bind to M protein (Table 2.2). These observations also imply that M protein contains at least two binding sites: one for actin, which is sensitive to iodination damage and another for carbonic anhydrase, which is not sensitive to iodination damage.

Bovine DNaseI has been shown to bind to actin with a K_D of 2×10^{-9} M (Mannherz *et al.*, 1980). In my study, I could not measure the binding constant of actin to DNaseI because of my inability to iodinate DNaseI using the Iodo-Gen method. In addition, ^{125}I-actin did not interact with DNaseI-coated polystyrene tubes. However, competitive binding assays

demonstrated that DNaseI, like carbonic anhydrase, did not compete with M protein for binding sites on ^{125}I-actin. In these studies I have assumed that DNaseI binds to uniodinated actin as reported by Mannherz *et al.* (1980).

DISCUSSION

Actin has been found in association with several viruses including paramyxoviruses. A possible role for actin microfilaments in the assembly and budding of paramyxoviruses has been proposed (Tyrrell and Ehrnst, 1979; Bohn *et al.*, 1986; Hambly *et al.*, 1986). Whether or not actin filaments play a role in viral assembly and budding, the binding of M protein to actin may explain the presence of cellular actin in the virion and may play an important role in the appearance of anti-actin antibodies in patients infected with paramyxoviruses. Antibodies to native actin are difficult to produce. Therefore, it is surprising that anti-actin antibodies are frequently present in the sera of patients following infections with paramyxoviruses. I believe the ability of these infections to induce anti-actin antibodies may be enhanced as a consequence of the association of M protein with cellular actin. This association could increase the immunogenicity of cellular actin, possibly through a change in the tertiary structure of the molecule and exposure of hidden antigenic sites to the host immune system.

An interaction between paramyxovirus membrane protein and cellular actin has previously been demonstrated (Giuffre *et al.*, 1982). Further studies using affinity chromatography and ELISA have now been completed in this study. The results show that removal of the N-terminus of the actin molecule

(amino acid residues 1-62) decreases the binding of actin to M protein. This suggests that the N-terminal 62 amino acids may be partially involved in the binding of M protein to actin or that removal of the N-terminal 62 amino acids results in some conformational change in the tryptic-resistant fragment which decreases the M protein binding to actin. I favor the latter explanation since it has been shown by Hambly *et al.* (1986) that DNaseI binds to residues 48 to 82 of actin. I have shown that the binding of DNaseI to actin did not interfere with the binding of M protein to actin.

M protein binds to actin with a high affinity. The measured dissociation constant was 6.3×10^{-8} M. This represents a binding affinity of 1.6×10^{7}/M. This association constant is in the same range as many antibody-antigen interactions (Sachs *et al.*, 1972). The confirmation of this relatively high binding affinity of M protein for actin supports our hypothesis that this is a significant biological interaction. This could be on the basis of an ionic interaction. M proteins are very basic with relatively high pI values whereas the actin molecule contains a number of negative charges, particularly in the N-terminal portion (Vandekerckhove and Weber, 1984). Against the possibility that this is just an ionic interaction is the fact that the interaction can occur in quite high salt concentrations. For example, the ELISA plate coated with actin can interact with

the M protein solution which was diluted with PBS as shown in Fig. 2.2.

The iodination of M protein reduced the binding of M protein to actin as shown in Table 2.2. This result suggests that the actin binding site of M protein or the flanking region of the binding site may contain tyrosine. Although this gives more insight into the M protein binding site on the actin molecule, further studies to localize the M protein binding site on actin and the effect of M protein binding on the conformation of actin will be required. The binding of M protein to carbonic anhydrase was a fortuitous observation. M protein binds to carbonic anhydrase with a lower dissociation constant (K_D=1.0x10^{-7} M). My results suggest that carbonic anhydrase and actin bind to M protein in different sites since carbonic anhydrase did not interfere with actin binding to M protein and actin did not interfere with carbonic anhydrase binding to M protein.

TABLE 2.1*

Actin monomer-sequestering proteins.

Protein	Molecular weight (kDa)	Binding affinity (Ka) (M^{-1})	Iso-electric pH	Source
Profilin	12-15	$0.05\text{-}2\times10^{7}$	9.1	Eukaryotic cells
Vitamin D-binding protein (G_c-globulin)	57	1.8×10^{7}	4.9	Blood plasma
Deoxyribo-nuclease I	31	5×10^{8}	4.7	Bovine pancreas
Depectin	19	2×10^{6}	6.1	Starfish oocytes
19 kDa protein	19	$\sim10^{6}$	6	Porcine brain

* Stossel *et al.*, 1985.

TABLE 2.2

Competitive binding assay of ^{125}I-actin to virus M protein-coated polystyrene tubes.

Inhibitor	Concentration [IC_{50}] resulting in 50% inhibition of specific binding of actin to M protein-coated tubes
Actin	1.14×10^{-6} M
M protein	1.14×10^{-6} M
Iodinated-M protein	2.70×10^{-6} M
DNaseI	$>1.0 \times 10^{-4}$ M
carbonic anhydrase	$>1.0 \times 10^{-4}$ M

Virus M protein-coated polystyrene tubes were incubated with 0.02 µM ^{125}I-actin in the presence of various concentrations (0.1 µM to 10 µM) of unlabelled actin, virus M protein, unlabelled iodinated virus M protein, DNaseI or carbonic anhydrase.

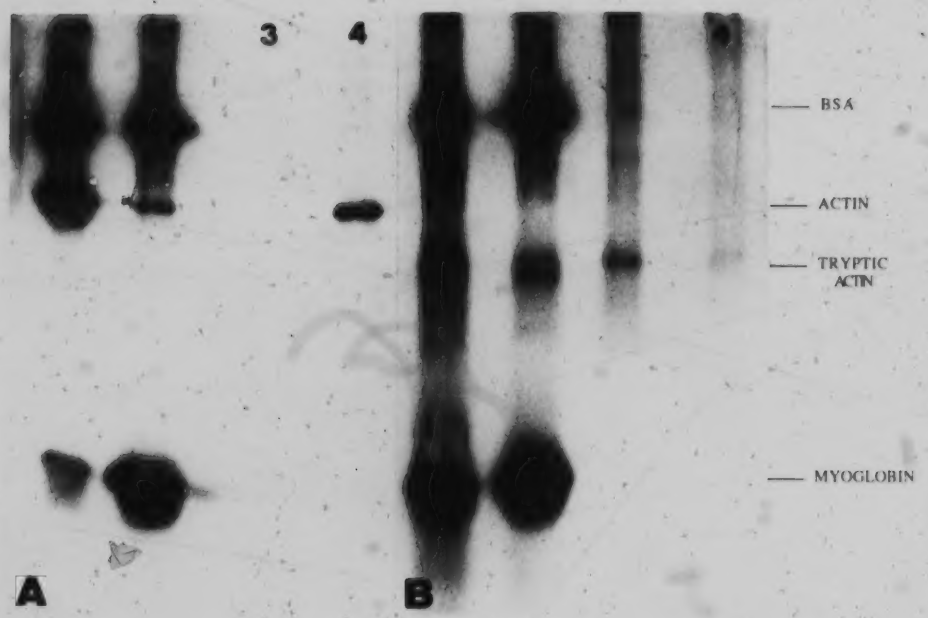


Figure 2.1 SDS-PAGE of protein applied to and eluted from affinity column of NDV M protein linked to Sepharose 4B. Mixtures of ^{125}I labelled proteins (actin, BSA and myoglobin in (A) and tryptic fragment of actin, BSA and myoglobin in (B) were loaded onto 3 ml columns. The protein mixtures were eluted with 10 ml of PBS, 10 ml of 2M KCl and 10 ml of 8 M urea sequentially and applied to SDS-polyacrylamide gels. After electrophoresis, the gels were exposed to X-ray film for autoradiography.

lane 1. protein in the original mixture.
lane 2. protein in the PBS eluate.
lane 3. protein in the 2 M KCl eluate.
lane 4. protein in the 8 M urea eluate.

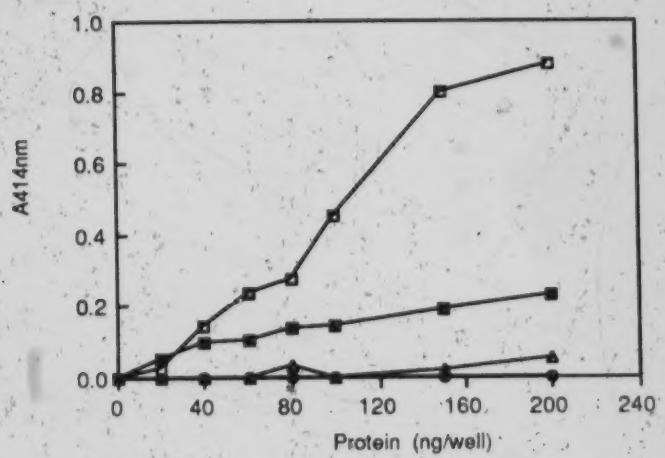


Figure 2.2 Interaction of M protein and actin as determined by ELISA. Micro ELISA plates were coated with various amounts of actin, tryptic fragment of actin, BSA, and myoglobin at 0 ng to 200 ng per well. The plates were incubated with 100 µl (1 mg/ml) of NDV M protein for 1 h at 37 °C followed by incubation with anti-M protein antibodies. Symbols: □, actin; ■, trypsin-digested actin fragment; Δ, myoglobin ; O, BSA.

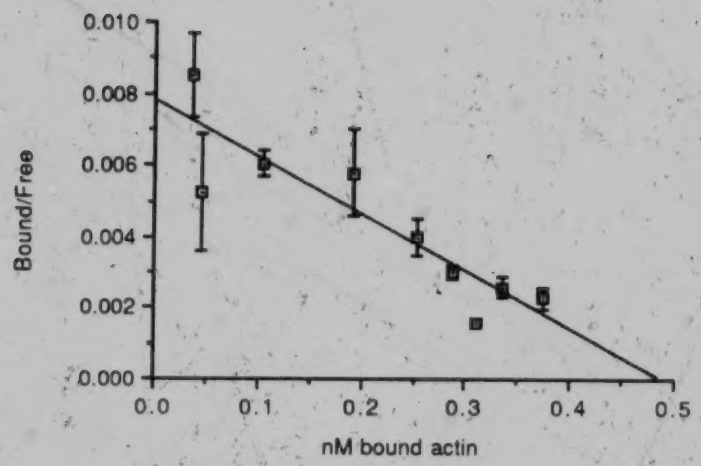


Figure 2.3 Scatchard analysis of actin-M protein binding data. Equilibrium binding assays were performed in 12x75 mm M protein-coated polystyrene tubes at room temperature. All assays were performed in triplicate and were incubated with or without excess unlabelled ligand (10 μM) to evaluate non-specific binding. The data points represent the average of triplicate determinations ± standard error of means. The binding of ^{125}I-actin to M protein produces a linear Scatchard plot with the goodness of fit equals to 0.798. Its slope is -1.56×10^{7} M^{-1} and is used to calculate K_D since the slope equals $-1/K_D$. The intercept on the abscissa equals the concentration of actin binding sites.

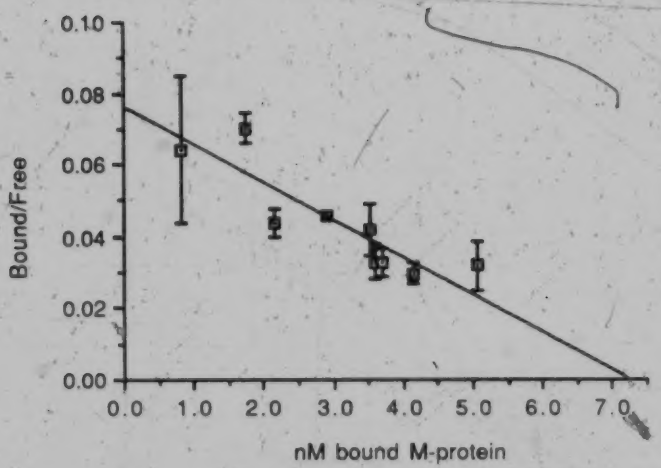


Figure 2.4 Scatchard analysis of carbonic anhydrase-M protein binding data. Unlabelled carbonic anhydrase coated polystyrene tubes were incubated with ^{125}I-labelled M protein (3.6 nm to 100 nm), with or without excess unlabelled ligand (10μM) as described in the legend to Fig. 2.3. All assays were performed in triplicate. The binding of ^{125}I-M protein to carbonic anhydrase produces a linear Scatchard plot with a slope of $-1x10^{7}$ M^{-1}, which is used to calculate K_D based on the formular that slope=$-1/K_D$. Its intercept on the abscissa equals to the concentration of M protein binding sites.

BIBLIOGRAPHY

Armstrong, G.D., and Peppler, M.S. 1987. Maintenance if biological activity of pertussis toxin radioiodinated while bound to fetuin-agarose. Infect. Immun. 55(5): 1294-1299.

Berzofsky, J.A.,Epstein, S.L., and Berkower, I.J. 1989.Antigen-antibodies interaction and monoclonal antibodies. In: Fundamental immunology, Paul, W. (ed.), pp.315-338, second edition. Raven Press. Ltd. , New York.

Bohn, W., Rutter, G., Hohenberg, H., Mannweiler, K., and Nobis, P. 1986. Involvement of actin filaments in budding of measles virus: studies on cytoskeletons of infected cells. Virology. 149: 91-106.

Boyer, M., Roustan, C., and Benyamin, Y. 1985. DNaseI actin complex: an immunological study. Bioscience Report. 5: 39-46.

Giuffre, R.M., Tovell, D.R., Kay, C.M.,, and Tyrrell, D.L.J. 1982. Evidence for an interaction between the membrane protein of a paramyxovirus and actin. J. Virol. 42(3): 963-968.

Goldschmidt-Clermont, P.J., Galbraith, R.M., Emerson, D.L., Marsot, F., Nel, A.E., and Arnaud, P. 1985. Distinct sites on the G-actin molecule bind group-specific component and deoxyribonuclease I. Biochem. J. 228:471-477.

Hambly, B.D., Barden, J.A., Miki, M., and dos Remedios, C.G. 1986. Structural and functional domains on actin. Bioessays. 4(3): 124-128.

Jacobson, G.R., and Rosenbusch, J.P. 1976. ATP binding to a protease-resistant core of actin. Proc.Natl.Acad. Sci. U.S.A. 73:2742-2746.

Lacks, S.A., 1981. Deoxyribonuclease I in mammalian tissues. J. Biol. Chem. 256(6): 2644-2648.

Lazarides, E., and Lindberg, U. 1974. Actin is the naturally occurring inhibitor of deoxyribonuclease I. Proc. Natl. Acad. Sci. U.S.A. 71: 4742-4746.

Mannherz, H.G., Goody, R.S., Konrad, M.,and Nowak, E. 1980. The interaction of bovine pancreatic deoxyribonuclease I and skeletal muscle actin, Eur. J. Biochem. 104: 367-379.

Scheid, A., and Choppin, P.W. 1973. Isolation and purification of envelope protein of Newcastle disease virus. J. Virol. 11: 263-271.

Spudich, J.A., and Watt, S. 1971. The regulation of rabbit skeletal muscle contraction. J. Biol. Chem. 246; 4866-4871.

Stossel, T.P., Chaponnier, C., Ezzell, R.M., Hartnig, J.H., Janmey, P.A., Kwiatkowski, D.J., Lind, S.E., Smith, D.B., Southwick, F.S., Yin, H.L., and Zaner, K.S. 1985. Nonmuscle actin-binding proteins. Ann. Rev. Cell.Biol. 1: 353-402.

Sutoh, K. 1984. Actin-actin and actin-deoxyribonuclease I contact sites in the actin sequence. Biochemistry. 23: 1942-1946.

Tyrrell, D.L.J., and Ehrnst, A. 1979. Transmembrane communication in cells chronically infected with measles virus. J. Cell Biology 81: 396-402.

Tyrrell, G.L., Peppler, M.K., Bonnah, R.A., Clerk, C.G., Chong, P, and Armstrong, G.D. 1989. Lectin like properties of pertussis toxin. Infect. Immun. 57(6): 1854-1857.

Vaananen, H.K., Timo, E.S., Tolonen, U., Vuori, J., Myllyla, V.V. 1988. Muscle specific carbonic anhydrase III is a more sensitive method of muscle damage than creatine kinase in neuromuscular disorders. Arch. Neurol. 45: 1254-1256.

Yoshida, T., Nagai, Y., Yoshi, S., Maneo, K., Mutsumoto, T., and Hashino, M. 1976. Membrane (M) protein of HVJ (Sendai virus): its role in virus assembly. Virology. 71: 143-161.

CHAPTER 3

Paramyxovirus M Protein Augments the Production of Anti-actin Antibodies[3]

An autoantigen may be protein, nucleic acid, phospholipid, lipoprotein, sugar or steroid. Autoantigens can be categorized into 2 groups: organ specific and systemic (ubiquitous) antigens. Autoantibodies are antibodies produced by humans or other animals which react with the antigens present in their own cells or extracellular proteins. Natural autoantibodies have been found in sera from normal animals including humans (Karsenti *et al.*, 1977; Guilbert *et al.*, 1982). Hybridomas obtained from normal mice or immune mice at different ages produced antibodies against various self antigens (Dighiero *et al.*, 1983; Dighiero *et al.*, 1985; Guilbert *et al.*, 1985). Antigen-presenting cells (APCs) from unimmunized mice can stimulate hybridomas specific to hemoglobin in the absence of exogenous hemoglobin. These results suggest that there is a constitutive processing and presentation of self molecules (Lorenz and Allen, 1988). These results also suggest that

---

[3] Information from this chapter has been published in the following papers: (1)Tyrrell, D.L.J.; Taechaudomtavorn, W., Lund, G.A., Tovell, D.R. 1987. In: Mahy and Kalakofsky (eds.) The biology of negative strand viruses. Elsevier Science Publisher B.V. (Biomedical Division). (2) Anomasiri, W.T., Tyrrell, D.L.J. 1988 Clin. Exp. Immunol. 74: 59-62. (3). Anomasiri, W.T., Tovell. D.R., Tyrrell, D.L.J. 1990 J. Virol.(in press).

the immune system of normal animals is able to recognize and respond to self antigens.

If the presence of autoantibodies is a normal phenomenon, then what is the difference between healthy individuals and those who suffer from autoimmune diseases? Mahana *et al.* (1989) suggested that the switching of autoantibody isotypes from polyspecific IgM isotypes with low avidity to specific IgG isotypes with high avidity may reflect the role of autoantigens in autoimmune response. On the other hand, Shoenfeld and Cohen (1987) speculated that the difference is determined by the level of autoantibodies. However, several studies have reported high titers of autoantibodies without any apparent disease, especially in the healthy family members of patients with autoimmune disease (Shoenfeld *et al.*, 1987). Despite increasing knowledge about autoantibodies, our understanding of some of the fundamental principles in autoimmune diseases remains incomplete.

Most clinical investigators believe that an infection can trigger an immune mediated tissue injury based on the observation that an infection sometimes precedes the development of an autoimmune disease. For instance, the onset of diabetes mellitus type 1 (IDDM) has been observed following mumps or coxsackie virus infection (Christiansen *et al.*, 1983). From serological studies there may be a relationship between cytomegalovirus infection and the

development of Sjogren syndrome in some patients (Shillitoe, 1982). High titers of measles virus antibodies have been reported in the sera of patients with chronic active hepatitis (Christie *et al.*, 1984). In this study the authors also detected the presence of autoantibodies reacting with actin. Infecting healthy animals with paramyxovirus resulted in the production of monoclonal antibodies to actin (Fagraeus *et al.*, 1983). Similarly, Hiruma and Hashimoto (1988) used the Sendai virus M protein to enhance the antigenicity of actin in the production of monoclonal antibodies. Thus, it is interesting to speculate on the relationship between paramyxovirus infection and the autoimmune response to actin.

The results from the previous chapter have demonstrated that paramyxovirus M protein interacts with cellular actin. This interaction may play a role in the production of anti-actin antibodies possibly through a conformational change of actin or by immune enhancement such as the provision of T-helper function for epitopes on actin. In this chapter I will examine the ability of M protein to augment the production of anti-actin antibodies in mice. I will also analyze the epitopes involved in the production of anti-actin antibodies in rabbits immunized with actin or actin plus M protein.

MATERIALS AND METHODS

Protein preparation. Actin was purified from acetone powders obtained from rabbit skeletal muscle by the method of Spudich and Watt (1971). Purified actin was digested with trypsin, thrombin and *Staphylococcus aureus* V-8 proteases according to published methods (Jacobson and Rosenbusch, 1976; Muszbek and Laki, 1974; Roustan et *al.*, 1985; respectively) prior to SDS-PAGE. Tryptic cleavage of actin yielded a major fragment comprising residues 63-375 (core) and small peptides (De Couet, 1983; Jacobson and Rosenbusch, 1976). Cleavage of actin by thrombin yielded the following peptides: 1-28 (I), 29-39 (II), 40-113 (M), 114-375 (L) and 40-375 (K) (Muszbek and Laki, 1974). *Staphylococcus aureus* V-8 protease cleaved actin into 2 fragments: 1-226 (N) and 227-375 (C) (Roustan et *al.*, 1985). After partial digestion with enzymes, actin fragments were electrophoresed on 10% SDS-PAGE and used for Western blot studies.

The M protein of Newcastle disease virus (NDV) was purified by the method of Scheid and Choppin (1973). Virus was disrupted with 2% Triton X-100 in 1 M KCl - 0.01 M sodium phosphate (pH 7.3) and insoluble proteins were removed by serial centrifugation at 10,000 x g for 30 min and 230,000 x g for 1 h. The M protein was precipitated from the supernatant by dialysis against a low salt buffer (0.01 M sodium phosphate, pH 7.3).

Bovine serum albumin (BSA), gelatin, bovine DNaseI and carbonic anhydrase were purchased from Sigma and their purity was confirmed by SDS-PAGE. They were used without further purification.

Mice and immunization. Inbred BALB/cCr mice, BALB/cCr nude mice and inbred C57Bl/10 mice were purchased from the Jackson Laboratory. BALB/cCr mice were immunized by foot pad injection with actin (50 µg) or actin (50 µg) mixed with 25 µg NDV M protein which was emulsified in an equal volume of complete Freund's adjuvant for a total volume of 50 µl per injection. The mice were given a boost of the same antigen in incomplete Freund's adjuvant one week after the initial immunization. A second boost with the same amount of soluble antigen in PBS was given intraperitoneally at 2 weeks after the primary immunization. The mice were bled from the tail vein four days after the second boost and sera were examined for anti-actin antibodies using an ELISA method (see below). Mice were immunized with 50 µg of gelatin or 50 µg of gelatin mixed with 25 µg of M protein. I also measured the antibody response to PPD, a protein in complete Freund's adjuvant. This antigen does not induce a strong B-cell response (like actin) and the effect of M protein on the production of an antibody response to PPD was studied. Ten animals were immunized with 50 µg of actin mixed with 25 µg of bovine serum albumin (BSA).

C57Bl/10 mice were immunized by foot pad injection with actin (50 µg); actin (50 µg) mixed with NDV-M protein (25 µg); actin (50 µg) mixed with DNaseI (25 µg); or carbonic anhydrase (50 µg) mixed with NDV-M protein (25 µg). All antigens were dissolved in the same manner and the animal immunizations were completed as described above with 50 µl injection.

Rabbits and immunizations. Actin (1.0 mg in G-actin buffer) was mixed with M protein (0.5 mg) and the solution left for 30 min at 22 °C prior to emulsification in complete Freund's adjuvant (CFA). Dutch rabbits were immunized by intramuscular injection and received booster injections of an equivalent amount of the proteins in incomplete Freund's adjuvant (IFA) at 4 and 5 weeks. Sera were taken at 6 weeks post immunization and tested for antibody production by ELISA and immunoblotting.

Actin (1.0 mg) was mixed with M protein buffer containing an amount of Triton X-100 equivalent to that in the M protein preparation. This was used in a similar immunization schedule to produce anti-actin antibodies. M protein (0.5 mg) was mixed with an equivalent amount of G-actin buffer and used to immunize Dutch rabbits in the same schedule as described above to produce anti-M protein antibodies. All injections were in a volume of 1 ml.

Enzyme linked immunosorbent assay (ELISA) For detection of anti-actin antibodies, micro-ELISA plates were coated with 100 ng of actin per well in phosphate buffered saline (PBS). After overnight incubation at 4 °C, the plates were washed with PBS containing 0.1% Tween 20 (PBS-Tween). Mouse sera were diluted 1:10, 100 µl was added to each well and incubated at 37 °C for 1 h. After washing the plates four times with PBS-Tween, 100 µl of peroxidase conjugated with rabbit anti-mouse immunoglobulin (Cappel Laboratories) was added and the plates were incubated at 37 °C for 1 h. The plates were washed 5 times with PBS-Tween, and 100 µl of H_2O_2 and 2,2'-azino, di [3-ethyl-benzthiazoline sulfonate] (ABTS) was added. Plates were incubated at 37 °C for 20 min and absorbance was read at 414 nm in a Titertrek multiscan photometer. The antibodies to PPD, gelatin and carbonic anhydrase were assayed using the same technique in which the micro ELISA plates were coated with 100 ng/well of the appropriate antigen.

Immunoblotting. Actin and actin fragments (products of partial digestion with proteases) were electrophoresed on SDS-PAGE (15% acrylamide). After electrophoresis, the proteins in the gel were electrophoretically transferred from the SDS-polyacrylamide gels to nitrocellulose paper (0.2 µm pore size) in transfer buffer (25 mM Trizma, 192 mM glycine, 20% (v/v) methanol) (Van Eldik and Wolchok, 1984). After transfer, the nitrocellulose paper was rinsed briefly in PBS

and then incubated in 0.3% (v/v) glutaraldehyde in fresh PBS for 30 min at room temperature. The nitrocellulose paper was rinsed briefly in PBS and the non-specific binding sites were blocked by soaking in 0.5% BSA in PBS-Tween at room temperature for 1 h. After washing three times in PBS-Tween (10 min each at room temperature), the paper was exposed to the rabbit antisera diluted 1:2000 in 0.5% BSA-PBS, for 1 h at room temperature. The paper was washed as above and incubated with a peroxidase-labelled IgG antibody for 1 h at room temperature. After washing as above, the paper was incubated with substrate solution (50 mg of 3,3'-diaminobenzidine (3,3',4,4'-tetraaminobiphenyl-tetrahydrochloride), 100 µl of 3% H_2O_2 in 100 ml of PBS) to visualize fragments binding anti-actin antibodies. To detect the total transferred protein, the nitrocellulose papers were stained with 0.5% (w/v) Amido Black 45% (v/v) methanol, 10% (v/v) acetic acid in water and destained in water.

RESULTS

Immune response to cellular actin in BALB/cCr mice immunized with actin alone is lower than in mice immunized with actin and NDV M protein. Seven of ten mice immunized with actin and NDV M protein produced high titers of antibodies to actin, whereas none of the ten animals immunized with actin alone produced anti-actin antibodies. On the other hand, M protein failed to enhance antibody production to gelatin or PPD. Mice immunized with actin mixed with BSA failed to produce anti-actin antibodies (Table 3.1). Mice injected with actin in one foot pad and M protein in another foot pad also failed to produce anti-actin antibodies. This suggests the enhancement of anti-actin antibody production depends on an interaction between actin and NDV M protein prior to the administration of the antigen complex.

Ten BALB/cCr nude mice were used to study the enhancement of anti-actin antibodies production by NDV M protein. None of five mice immunized with actin alone produced anti-actin antibodies. Only one of five nude mice immunized with actin and NDV M protein produced a low titer of anti-actin antibodies as shown in Table 3.2. Therefore, the ability of NDV M protein to enhance anti-actin antibody production appears to be associated with thymus-derived lymphocytes.

Antisera from mice immunized with actin or carbonic anhydrase alone were compared with antisera from mice immunized with actin-M protein and DNaseI-M protein or carbonic anhydrase-M protein complexes. Only mice immunized with the actin-M protein complex produced anti-actin antibodies, whereas mice immunized with actin alone or actin mixed with DNaseI did not produce anti-actin antibodies (Fig. 3.1). Mice immunized with carbonic anhydrase alone produced antibodies to this enzyme, but the production of antibodies was not enhanced when the mice were inoculated with carbonic anhydrase-M protein complexes (Fig. 3.2).

Mapping of new antigenic determinant(s) on actin molecule. Five Dutch rabbits were immunized with undenatured actin alone and all produced antibodies to actin. However, none produced antibodies to the 33 kDa core fragment after digestion of actin with trypsin. Five animals were immunized with a mixture of actin and M protein, and all produced antibodies to both undenatured actin and to the core fragment. Antisera from these animals were tested in immunoblots against a number of other fragments of actin in order to determine the number and location of any new antigenic determinants. The actin peptides were obtained by digestion with several proteases known to cleave actin at specific sites (Fig. 3.3). The antibodies obtained from rabbits immunized with actin alone react with the whole actin molecule and with the N-terminal peptide from *S. aureus* V-8

protease digestion (Fig. 3.4, panel c). Anti-actin antibodies produced by animals immunized with actin-M protein complex reacted with the whole actin molecule, with both the N (1-226) and C (227-375) peptides from *S. aureus* V-8 protease digestion, with the K (40-375), L (114-375), M (40-113) peptides from thrombin digestion and also with an extra peptide (114-226) obtained from a double digestion with thrombin and *S. aureus* V-8 protease (Fig. 3.4, panel d). These results support our hypothesis that upon the interaction of actin with M protein, there is a conformational change in the tertiary structure of actin that allows new antigenic determinants to be exposed to the immune system.

The relevance of this study to post-viral autoimmune responses to actin is unknown. However, when four samples of human serum were tested for anti-actin antibodies by immunoblotting, two of three sera from patients who had recovered from acute measles infections, and thus had anti-measles antibodies, also had antibodies to both actin and the 33 kDa actin fragment. There was no evidence for anti-actin antibodies in the third serum nor in a fourth serum sample taken from a patient without measles antibodies (Fig. 3.5). The results of this small study confirm the presence of anti-actin antibodies following measles infection, as previously reported (Haire, 1972), and provide some support for the use

of our experimental system as a model to study the events of a natural infection.

The M protein binding site and the antigenic determinant on the actin molecule are different. Dutch rabbits were immunized with actin or M protein. The antisera in these rabbits were collected at 6 weeks post-immunization. Micro-ELISA plates were coated with 100 ng of actin per well. After incubation overnight, the plates were washed and incubated with various amounts of M-protein (0-200 ng per well) for 1 h at 37 °C. The plates were washed again and incubated for 1 h at 37 °C with a 1:2000 dilution of each rabbit antiserum produced against actin or M protein. The ELISA was completed as described above. When increasing amounts of M protein were bound to the actin there was increased binding of anti-M protein antibodies. The addition of M protein to the actin-coated plate did not decrease the binding of anti-actin antibodies. Since the binding of anti-actin antibodies to native actin was not decreased in the presence of increasing amounts of M protein, this suggested that the binding site of M protein to actin was distinct from the antigenic site in native actin (Fig. 3.6b). The converse of this experiment was done with M-protein coating of the ELISA plate and the addition of increasing amounts of actin. The increase in binding of anti-actin antibodies was consistent with binding of actin to M protein (Fig. 3.6a). The failure of actin to decrease the binding of anti-M

protein antibodies to M protein was in keeping with actin binding to M protein at a site different from the antigenic site on M protein. This result also confirmed that there was no cross-reactivity between antisera for actin and M protein.

Micro-ELISA plates were coated with 100 ng of the N fragment of actin per well. After incubation overnight, the plates were washed and incubated with various amounts of M-protein (0-200 ng per well) for 1 h at 37 °C. The plates were washed again and incubated for 1 h at 37 °C with a 1:2000 dilution of each rabbit antiserum produced against actin, or M protein. The ELISA was completed as described above. When increasing amounts of M protein were bound to the N fragment of actin on the plates, increased binding of anti-M protein antiserum, but not anti-actin antiserum was observed (Fig. 3.7). Since the binding of anti-actin antibodies to native actin is not decreased by increasing amounts of M protein, this further suggested that the binding site of M protein to actin was different from the antigenic site in native actin and that it was located in the N-terminal fragment of actin (amino acids residue 1-226). This is consistent with our previous finding that the trypsin resistant actin fragment (63-375) has decreased binding to M protein compared to native actin.

DISCUSSION

The role of viral infection in autoimmunity and the possible mechanisms of viral-induced autoimmune responses has been summarized by Notkins *et al.* (1984). We have no evidence of "antigenic mimicry" in our system since antibodies to M protein do not recognize any antigenic sites on actin (Fig. 3.6). Viral antigens can act as helper determinants for the antibody response to cell surface antigens as demonstrated by Bromberg *et al.* (1982). In the system described by Bromberg *et al.*, the augmentation appears to be mediated by a carrier-hapten effect; however, the nature of the viral antigen which may interact with the host cell antigen (thy-1) remains unclear.

Previous studies have demonstrated that the M protein of paramyxoviruses interacts with actin (Giuffre *et al.*, 1982). In the present study I have shown that paramyxovirus M protein augments the immune response to cellular actin. This response requires the physical interaction between M protein and actin since immunization with these antigens in separate sites fails to induce antibodies to actin. One possible mechanism for the augmentation is that M protein interacts with actin and this physical association results in a carrier-hapten effect with M protein being the helper antigen recognized by sensitized T-cells. The mechanism of augmentation by the viral protein in terms of a carrier

effect should be thymus dependent. The failure of M protein to augment the immune response to actin in nude mice is consistent with this hypothesis. I believe that this study is unique since I have identified a viral antigen which interacts with a specific host cell protein to induce an autoimmune response. This may explain the frequent presence of anti-actin antibodies following acute infection with paramyxoviruses (Fagraeus and Norberg, 1978). It also supports the observation of virus-induced autoimmune responses by a linked carrier effect. Another possiblity is that the M protein induces an alteration in the tertiary structure of actin which results in new epitopes in this host protein. This could result in altered processing by antigen presenting cells.

Actin interacts with a wide range of proteins in addition to self-assembling into filaments. The binding sites of several proteins on the actin molecule have been localized. The myosin heavy chain binds to residues 1-12 in the N-terminal region, the myosin light chain binds at or very near residues 360-373 in the C-terminal region, and DNaseI binds to residues 48-82 (Hambly *et al.*, 1986). M protein specifically binds to actin or the tryptic fragment of actin (residues 63-375). Preliminary results from the previous chapter based on ELISA studies with the actin core fragment (residues 63-375) (Fig. 2.4) and in this chapter using the N-terminal fragment (residues 1-226) (Fig. 3.7)

would suggest that the M protein binding site is between residues 63-226 but further work is required to confirm this location.

Two antigenic determinants have been identified in the N-terminal region of the native actin molecule corresponding to the sequences between amino acid residues 1-7 and 18-28 (Roustan *et al.*, 1986). Either one or both of these antigenic determinants are the sites responsible for anti-actin antibody production with undenatured actin. Since the epitope at residues 1-7 is species-specific (I used rabbit actin in rabbits), the epitope most likely stimulating the immune system in rabbits immunized with rabbit skeletal muscle actin would be the epitope between residues 18-28, the conserved sequence which is known to be antigenic (Mejean *et al.*, 1987). If M protein is allowed to interact with actin before immunization, the resulting antiserum reacts with at least 3 new epitopes located in fragments 40-113, 114-226 and 227-375 of the actin molecule. Parker *et al.* (1986) developed a computerized prediction of antigenic determinants using a combination of three parameters (HPLC hydrophilicity, Janin accessibility and Karplus and Schultz flexibility) to generate a composite surface profile. Considering the primary structure of the actin molecule, the probable antigenic sites predicted with the Parker program were located at amino acid residues 56-64, 113-120, 150-160, 330-338 and 356-368. This prediction is consistent with the new

antigenic determinants demonstrated after the binding of M protein to the actin molecule.

Previous studies have shown that M protein binding to actin induces a conformational change (Giuffre *et al.*, 1982) and I have shown that interaction with M protein enhances antibody production to new antigenic determinants in the actin molecule. We postulate that the "new" antigenic determinants are normally unrecognized by the immune system. This is an example in which a viral protein interacts with a cellular protein to produce new epitope(s). This effect could be explained by an alteration in the tertiary structure of the host cell protein to expose new epitope(s). Alternatively, the M protein could enhance the T-helper cell response through a carrier effect or produce specific T-cell help for the antibody response to new actin epitope(s). It is also possible that M protein interaction with actin alters the antigen processing by antigen presenting cells to enhance the immune response to actin.

TABLE 3.1

Enhancement of antibody production in BALB/cCr mice by NDV M protein

Immunogen	Antigen used in ELISA	Antibody responses measured by ELISA: Number of positive sera	Antibody responses measured by ELISA: Mean A_{414nm} ± S.D.
Actin	Actin	0/10	0.066±0.031
A,M[1]	Actin	0/10	0.083±0.035 (N.S.)[3]
A+M[2]	Actin	7/10	0.332±0.238 ($p<0.025$)
Actin+BSA	Actin	1/10	0.164±0.118 (N.S.)
M protein	M protein	10/10	0.687±0.107
M protein	Actin	0/10	Non-reactive
PPD	PPD	0/10	Non-reactive
PPD+M	PPD	0/10	Non-reactive
Gelatin	Gelatin	14/14	0.148±0.047
Gelatin+M	Gelatin	15/15	0.233±0.136 (N.S.)

(1) Mice immunized with actin and NDV M protein in different foot pads.

(2) Mice immunized with actin incubated with M protein.

(3) Significant difference calculating by student-t-test, (N.S.=not significant).

TABLE 3.2

Immune response to actin alone and actin and NDV M protein in BALB/cCr and BALB/cCr (nude) mice

Immunogen	Number of mice producing anti-actin antibodies	
	BALB/cCr	BALB/cCr (nude)
Actin	0/10	0/5
Actin+NDV-M protein	7/9(1)	1/5(2)

(1) Mean A_{414} ± S.D. in responders (n=7) = 0.74±0.40.

(2) A_{414nm} in the one responder =0.352.

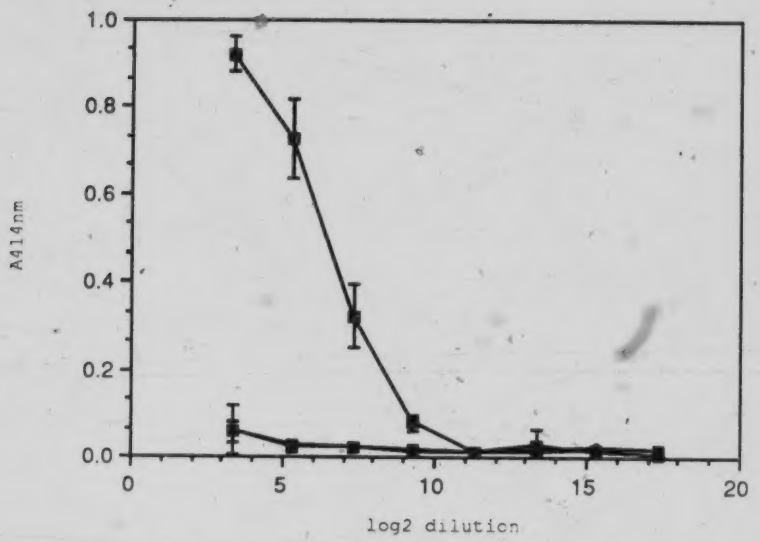


Figure 3.1 Effect of M protein and DNaseI on actin antibody production. The antibody titers were measured by the ELISA procedure using a peroxidase conjugated anti-mouse IgG. Micro-ELISA plates were coated with 100 ng of actin in PBS per well. The plates were incubated with antisera from mice immunized with actin (50 µg) in buffer (O); actin (50 µg) mixed with M protein (25 µg) (□); or actin (50 µg) mixed with DNaseI (25 µg) (■). Groups of mice (n=5) were used in this study. All animals received a primary immunization by foot pad inoculation with antigen in complete Freund's adjuvant and received a boost at day 10 with the same antigen in incomplete Freund's adjuvant. Antibody titers were measured 17 days after the primary immunization.

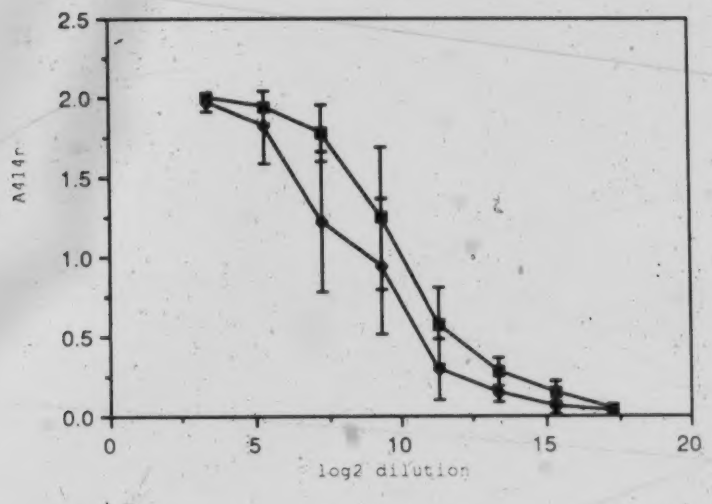


Figure 3.2 Effect of M. protein on carbonic anhydrase antibody production. The antibody titers were measured by the ELISA procedure using a peroxidase conjugated anti-mouse IgG. Micro-ELISA plates were coated with 100 ng of carbonic anhydrase. The plates were incubated with antisera from mice immunized with carbonic anhydrase (50 µg) mixed with M buffer (□); or carbonic anhydrase (50 µg) mixed with M protein (25 µg) (♦). Groups of mice (n=5) were used in this study. All animals received a primary immunization by foot pad inoculation with antigen in complete Freund's adjuvant and received a boost at day 10 with the same antigen in incomplete Freund's adjuvant. Antibody titers were measured 17 days after the primary immunization.

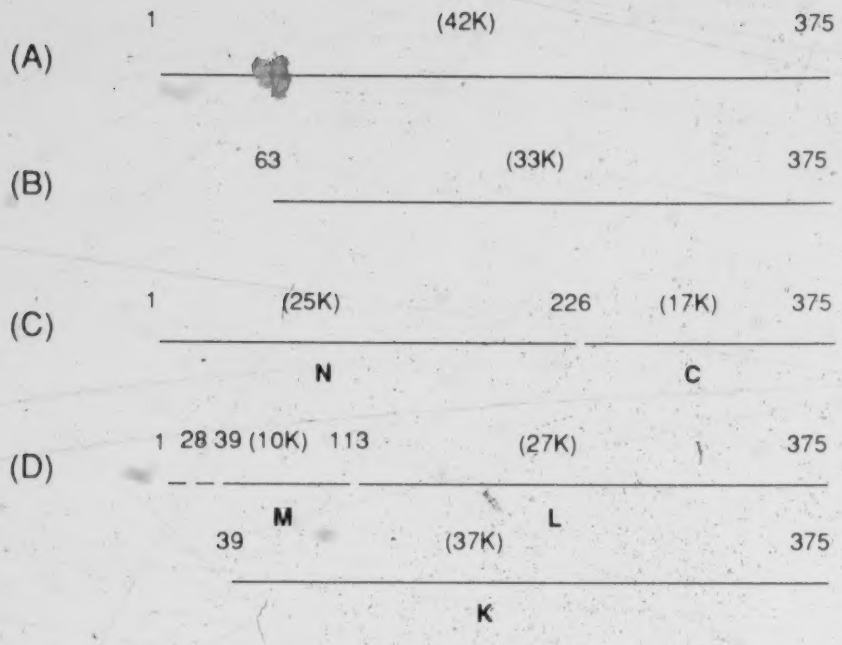


Figure 3.3 Actin fragments visible in SDS-PAGE gels after protease digestion.

(A) Undigested actin;

(B) Trypsin digested actin, 33 kDa fragment;

(C) *Staphylococcus aureus* V-8 protease digested actin, 25 kDa and 17 kDa fragments;

(D) Thrombin digested actin, 10 kDa, 27 kDa and 37 kDa fragments.

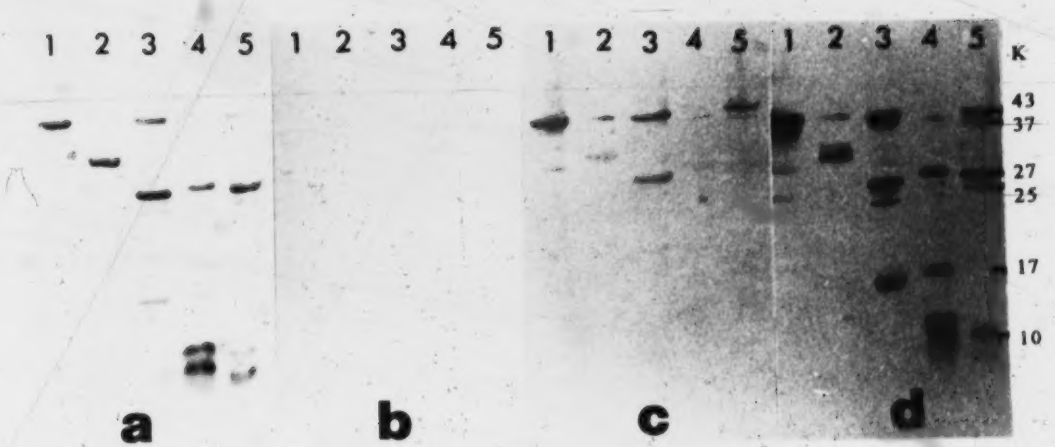


Figure 3.4 Actin mapping. Proteins were electrophoresed on SDS-PAGE (15% acrylamide) and electrotransferred onto nitrocellulose membranes. The membranes were incubated for immunoblotting with sera from rabbits: (a) amido black staining of the original blot after eletrotransferring, (b) preimmunization, (c) immunized with actin, and (d) immunized with actin and M protein.

lane 1. undigested actin.
lane 2. trypsin digested actin.
lane 3. *S. aureus* V-8 protease digested actin.
lane 4. thrombin and *S. aureus* V-8 protease digested actin.
lane 5. thrombin digested actin.

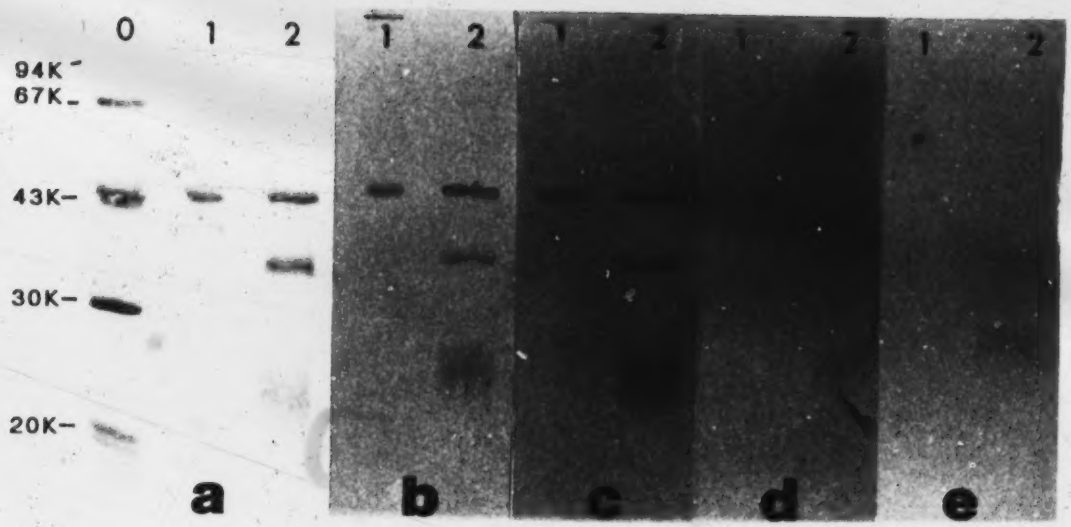


Figure 3.5 Western blotting results for human antisera containing measles antibodies and/or anti-actin antibodies. Panel a. shows amido black -B staining of marker proteins. Panels b. and c. show Western blotting of human sera, from patients convalescent for measles infections, containing actin antibodies; panel d. human serum containing measles antibodies but no anti-actin antibodies; panel e. human serum without antibodies to measles or actin.

lane 0. marker proteins.
lane 1. undigested actin.
lane 2. trypsin-digested actin.

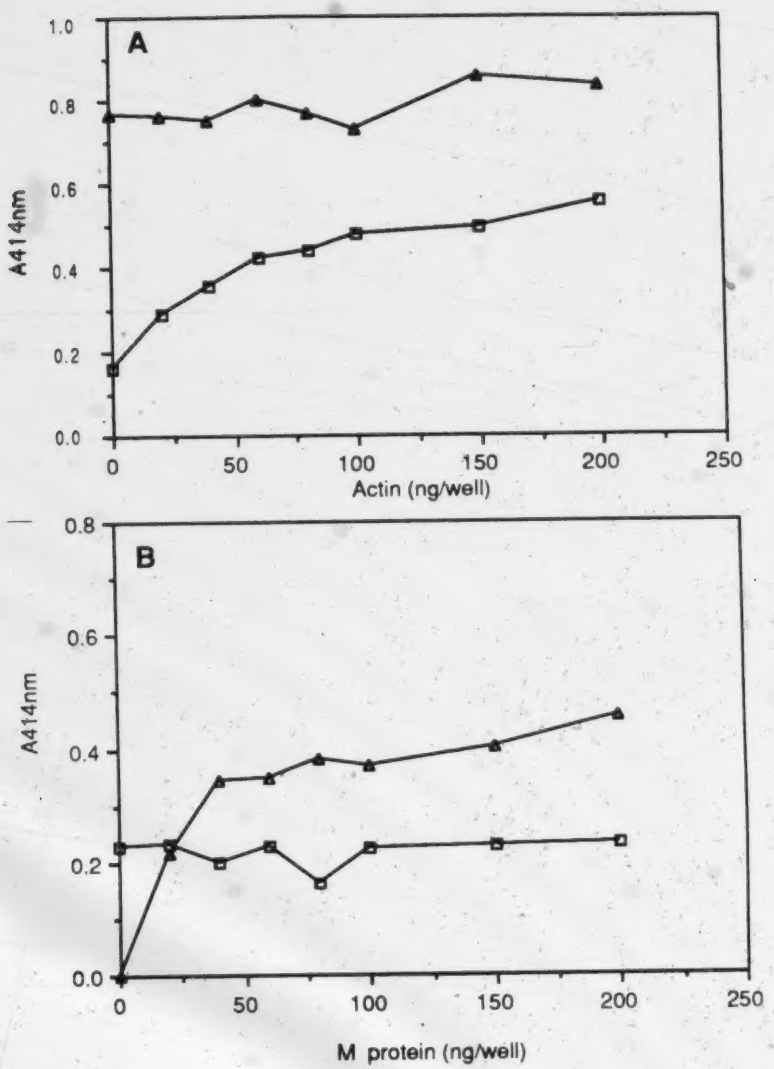


Figure 3.6 The membrane protein of NDV and anti-actin antibodies bind to different sites on actin. In panel A, each well of an ELISA plate was coated with 100 ng of M protein and actin in increasing amounts was added to the wells. In panel B, each well of an ELISA plate was coated with 100 ng of actin and M protein in increasing amounts was added to the wells. The plates were incubated with rabbit antisera to actin (□) or rabbit antisera to M protein (Δ).

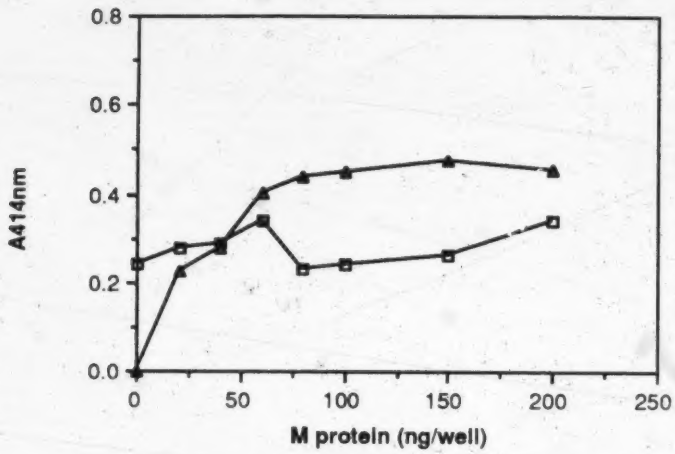


Figure 3.7 ELISA determination of M protein binding to N fragment of actin cleaved by *S. aureus* V-8 protease. Micro ELISA plates were coated with 100 ng of N fragment of actin per well. The plates were incubated with anti-actin antibodies (□) and anti-M protein antibodies (Δ).

BIBLIOGRAPHY

Bromberg, J.S., Lake, P., and Brunswick, M. 1982. Viral antigens act as helper determinants for antibody response to cell surface antigens. J. Immunol. 129: 683-688.

Christiansen, M.L., Pachman, L.M., Maryjowski, M.C., and Friedman, J.M. 1983. Arthritis. Rheum. 26(suppl): S24

Christie, K.E., Endresen, C., and Hauekenes, G. 1984. IgM antibodies in sera from patients with chronic active hepatitis reacting with the measles virus matrix protein and nucleoprotein. J. Med. Virol. 14:149-157.

De Couet, H.G. 1983. Studies on the antigenic sites of actin: A comparative study of the immunogenic cross-reactivity of invertebrate actins. J. Muscle Res. Cell. Motil. 4: 405-427.

Dighiero, G., Lymberi, P., Holmberg, D., Lundquist, I., Coutinho, A., and Avrameas, S. 1985. High frequency of natural antibodies in normal newborn mice. J. Immunol. 134: 765-771.

Dighiero, G., Lymberi, P., Mazie, J.C., Rouyre, S., Butler-Brown, G.S., Whalen, R.G., and Avrameas, S. 1983. Murine hybridomas secreting natural monoclonal antibodies reacting with self antigens. J. Immunol. 131: 2267-2272.

Fagraeus, A., and Norberg, R. 1978. Anti-actin antibodies. Curr. Top. Microbiol. Immunol. 82: 1-13.

Fagraeus, A., Orvell, C., Norberg, R., and Norrby, E. 1983. Monoclonal antibodies to epitopes shared by actin and vimentin obtained by paramyxovirus immunization. Exp. Cell. Res. 145: 425-432.

Giuffre, R.M., Tovell, D.R., Kay, C.M., and Tyrrell, D.L.J. 1982. Evidence for an interaction between membrane protein of a paramyxovirus and actin. J. Virol. 42: 963-968.

Guilbert, B., Dighiero, G., and Avrameas, S. 1982. Naturally occurring antibodies against nine common antigens in normal human sera. I. Detection, isolation, and characterization. J. Immunol. 128: 2779-2787.

Guilbert, B., Mahana, W., Gilbert, M., Mazie, J.C., and Avrameas. S. 1985. Presence of natural autoantibodies in hyperimmunized mice. Immunology. 56: 401-408.

Haire, M. 1972. Fibrillar anti-cellular antibody associated with mumps and measles infection. Clin. Exp. Immunol. 12: 335-341.

Hiruma, S., and Hashimoto, S. 1988. Monoclonal antibody to rat brain actin antigenically enhanced with HVJ (Sendai virus) M protein. Histochemistry 90: 1-8.

Hambly, B.D., Barden, J.A., Miki, M., and dos Remedios, C.G. 1986. Structural and functional domains on actin. Bioessays. 4(3): 124-128.

Jacobson, G.R., and Rosenbusch, J.P. 1976. ATP binding to a protease-resistant core of actin. Proc.Natl.Acad. Sci. U.S.A. 73:2742-2746.

Karsenti, E., Guilbert, B., Bornens, M., and Avrameas, S. 1977. Antibodies to tubulin in normal non immunized animals. Proc. Natl. Acad. Sci. U.S.A. 74: 3997-4001.

Lorenz, R.G., and Allen, P.M. 1988. Direct evidence for functional self protein/Ia molecule complexes *in vivo*. Proc. Natl. Acad. Sci. U.S.A. 85: 5220-5223.

Mahana, W., Guilbert, B., and Avrameas, S. 1989. Studies on active immunization with self antigens. I. Production of antibody to unmodified proteins by neonatal immunization. Scand. J. Immunol. 30:295-302.

Mejean, C., Roustan, C., and Benyamin, Y. 1987. Anti-actin antibodies, detection and quantitation of total and skeletal muscle actin in human plasma using a competitive ELISA. J. Immunol. Method 99: 129-135.

Muszbek, L., and Laki, K. 1974. Cleavage of actin by thrombin. Proc. Natl. Acad. Sci. U.S.A. 71: 2208-2211.

Notkins, A.L., Onodera, T. and Prabhakar, B. 1984. Virus-induced autoimmunity. In Concepts in viral pathogenesis, pp. 210-215. Edited by Notkins, A.L. & Oldstone, M.B.A. Vienna & New York, Springer, Verlag.

Parker, J.M.R., Guo, D., and Hodges, R.S. 1986. New hydrophilicity scale derived from high-performance liquid chromatography peptide retention data: correlation of predicted surface residues with antigenicity and X-ray derived accessible sites. Biochemistry 25:5425-5432.

Roustan, C., Benyamin, Y., Boyer, M., and Cavadore, J.C. 1986. Structural variations in actins, a study of the immunological reactivity of the N-terminal region. Biochem. J. 233: 193-197.

Roustan, C., Benyamin, Y., Boyer, M., Bertrand, R., Audemard, E. and Jaurequi-Adell, J. 1985. Conformational changes induced by Mg^{2+} on actin monomers, an immunologic attempt to localize the affected region. FEBS Lett. 181: 119-123.

Scheid, A., and Choppin, P.W. 1973. Isolation and purification of envelope protein of Newcastle disease virus. J. Virol. 11: 263-271.

Shillitoe, E.J., Daniels. T.E., Whitcher, J.P., Vibeke-Strand, C., Tala, N., and Greenspan, J.S. 1982. Antibodies to cytomegalovirus in patients with Sjogren's syndrome. As determined by an enzyme-linked immunosorbent assay. Arthritis. Rheum. 25: 260-265.

Shoenfled, Y., and Cohen., I.R. 1987. Infection and autoimmunity. The antigens, 7: 307-325.

Shoenfeld, Y., Segol, G., Segol, O., Neary, B., Klajman, A., Stollar, B.D., and Isenberg, D.A. 1987. Detection of antibodies to total histones and their subfractions in systemic lupus erythematosus patients and their relatives. Arthritis. Rheum. 30: 169-175.

Spudich, J.A., and Watt, S. 1971. The regulation of rabbit skeletal muscle contraction. J. Biol. Chem. 246: 4866-4871.

Van Eldik, L.J., and Wolchok, S.R. 1984. Condition for reproducible detection of calmodulin and S100β in immunoblots. Biochem. Biophys. Res. Commun. 124: 752-759.

CHAPTER 4

Mechanism of Virus M Protein Induced Autoimmunity to Actin[4]

An autoimmune response may be one of the consequences of a viral infection. The mechanism by which a viral infection can induce an autoimmune response is frequently unknown. Several major mechanisms have been proposed including molecular mimicry, the production of anti-idiotype antibodies, a disturbance in the host immune response or a change in endogenous antigens (see pp. 26-32).

Evidence to support each of these proposed mechanisms has been reported. An autoimmune response may occur by molecular mimicry when a viral protein shares epitopes with host cell proteins (Oldstone, 1987; Oldstone and Notkins, 1986). For example, it has been shown that a number of viral proteins contain amino acid sequences homologous with the encephalitogenic site of the rabbit myelin basic protein (Fujinami and Oldstone, 1985). Furthermore, molecular mimicry is implied by the fact that 4% of monoclonal antibodies raised against RNA and DNA viruses cross-react with uninfected host cell tissues (Oldstone, 1987). Anti-idiotypic antibodies to viral antibodies may be specific for receptors on the cell surface. Krah and Choppin (1988)

---

[4] A version of this chapter has been prepared for submission to Journal of Immunology.

demonstrated that mice immunized with measles virus developed antibodies to a cell surface receptor for the virus. This mechanism has been implicated in several autoimmune diseases (Nepom *et al.*, 1982). Persistent viral infection can deregulate the normal host immune response and result in autoimmunity. This was shown in mice infected with lactate dehydrogenase elevating virus where polyclonal activation of immunoglobulin synthesis resulted in the production of autoantibodies (Cafruny *et al.*, 1986; Cafruny and Hovinen, 1988). Virus infection can promote the presentation of endogenous antigen to the immune system, possibly by interacting with the host cell proteins in such a way as to make them appear foreign to the host (Eaton, 1980).

Antigen processing and presentation

Class I versus class II pathway of antigen processing

The antigen receptors on T lymphocytes do not recognize free antigen, but rather foreign antigen in association with products of the major histocompatibility complex antigens (MHC). Table 4.1 summarizes the properties of class I and class II MHC antigens. Both class I and class II MHC molecules can serve as associative recognition structures in T-lymphocyte recognition of protein antigens. Cytotoxic T cells are usually induced by a viral antigen being expressed on the surface of infected cells in association with class I

antigens whereas helper T cells respond to soluble viral antigens expressed in association with class II antigens. The classification of antigenic presentation systems correlates fairly well with the division into endogenously synthesized antigens (cytotoxic T cells) versus exogenously introduced soluble proteins (T helper cells and antibody production). There is evidence to support the idea that there are two separate pathways for antigen processing and presentation associated with the two classes of MHC molecules (Braciale *et al.*, 1987). The two pathways can be differentiated by their sensitivities to different reagents. The class II pathway for soluble antigens is sensitive to chloroquine, a weak base (Morrison *et al.*, 1986). The class I-restricted or endogenous pathway is inhibited by the fungal antibiotic brefeldin-A (BFA), which blocks protein transport from the endoplasmic reticulum to the Golgi network (Lippincott-Schwartz *et al.*, 1989).

The endogenous pathway preferentially triggers a response from class I-MHC-restricted T lymphocytes directed to that antigen. Exogenous antigens usually fail to enter the class I pathway. However, direct introduction of the same proteins into the cell cytoplasm can result in class I-restricted T lymphocyte recognition (Moore *et al.*, 1988). Osmotic lysis of pinocytic vesicles containing native ovalbumin has been shown to sensitize EL4 target cells for cytotoxic T cell recognition, whereas cells incubated with

native ovalbumin did not become sensitized for recognition and lysis. Therefore, ovalbumin introduced by pinocytosis into the cytoplasm of target cells may gain access to a cytoplasmic degradation system prior to association with a class I molecule and presentation at the cell surface.

The processing events can be bypassed by use of synthetic peptides (Townsend and Bodmer, 1989; Brett *et al.*, 1988). Furthermore, identical immunogenic peptides can stimulate either class I-restricted cytotoxic T cells or class II-restricted helper T cells (Perkins *et al.*, 1989). The MHC class II-restricted helper T cells are composed of different $CD4^+$ subsets which produce different types of lymphokines. Th1 cells produce interleukin 2 (IL2) and interferon (IFN) which subsequently activate the cytotoxic T cells, whereas Th2 cells produce interleukin 4 (IL4) which specifically activates helper T cells (Murray *et al.*, 1989). The immune response is not only dependent on the intracellular pathway but also on the way the particular antigen is processed and the subsequent lymphokine response (Germain, 1986; Nuchtern *et al.*, 1990).

The specific interaction of actin and viral M protein enhances the production of anti-actin antibodies. The present study is concerned with the way in which a virus infection could change an endogenous (host cell) protein to render it immunogenic. I will present evidence that production of antibodies to native actin is controlled by MHC

gene products. T-cell proliferation assays were also performed to investigate the possibility that the enhanced immune response to actin in the presence of virus M protein was a result of T helper function. From these studies I believe there is evidence of altered antigenic processing in the presence of M protein that may result in enhanced anti-actin antibody production.

MATERIALS AND METHODS

Animals. C57Bl/10J and congenic strain B10.BR, B10.AKM, B10.A(4R), B10.A(5R), B10.D2, A.TH and A.TL mice, 4 to 6 weeks old, were obtained from the Jackson Laboratory, Bar Harbor, ME.

Immunization procedure. Rabbit skeletal muscle actin was purified by the method described previously by Spudich and Watt (1971). Paramyxovirus M protein was isolated from Newcastle disease virus as described by Scheid and Choppin (1973). Mice were immunized by foot pad injection with 50 µl of actin (50 µg) or actin (50 µg) mixed with NDV-M protein (25 µg) emulsified in an equal volume of complete Freund's adjuvant (CFA). The mice were given a booster injection with the same antigen in incomplete Freund's adjuvant (IFA) by food pad injection 10 days after the initial immunization. Mice were bled from the tail vein 4 days after the boost, and the sera were analyzed for the presence of anti-actin antibodies by ELISA. Five mice were immunized in each experimental group.

Enzyme linked immunosorbent assay (ELISA). For the detection of antibodies to actin, micro-ELISA plates were coated with 100 ng of actin per well in PBS. After overnight incubation at 4 ^{o}C, the plates were washed with PBS containing 0.1% Tween 20 (PBS-Tween). Sera were serially diluted, and 100 µl of each dilution was added to the

appropriate wells in the microtiter plates. The plates were incubated at 37 °C for 1 h. The plates were rinsed four times with PBS-Tween, then 100 µl of peroxidase conjugated anti-mouse immunoglobulin (Cappel Laboratories) were added to each well, and the plates were incubated at 37 °C for 1 h. The plates were washed with PBS-Tween five times, and 100 µl of H_2O_2 and 2,2'-azino, di [3-ethyl-benzthiazoline sulfonate] (ABTS) was added. Plates were incubated at 37 °C for 20 min and the optical absorbance was measured at 414 nm in a Titertek multiscan photometer.

T cell proliferation assay. Five mice were immunized with actin (50 µg) or actin (50 µg) mixed with NDV-M protein (25 µg) as described above. The mice were killed 10 days after immunization and their lymph nodes were aseptically removed. The lymph nodes were disrupted and suspended in a RPMI 1640 medium with 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 50 U penicillin/ml, 50 µg streptomycin sulfate/ml, and 0.01 M HEPES. The cells were washed once with medium and passed through a nylon wool column. The recovery of input cells was between 20 and 30% of the starting population (Lee *et al.*, 1979). The cells were washed twice with medium, counted and diluted to a final density of 2×10^6 cells/ml. Each microculture well contained 4×10^5 purified T cells and 1×10^6 irradiated syngenic spleen cells in a volume of 200 µl of medium containing antigen at concentrations of 1.25, 2.00, 5.0,

10.0, 20, 40, and 80.0 µg/ml. The control microculture wells contained cells but no antigen. Cultures were incubated for 4 days at 37 °C in an atmosphere of 5% CO_2 in humidified air. On the fourth day the cultures were radiolabelled with 1.0 µCi of [^{3}H]-thymidine (5 mCi/ml New England Nuclear Corporation, Boston, MA) overnight, harvested on fiberglass filter paper, and the radioactivity measured in a liquid scintillation counter. Results were expressed as means of triplicate samples.

RESULTS

Immune response to actin in actin primed mice. Actin, a highly conserved cytoskeletal protein, is a weak antigen. However, anti-actin antibodies have been found in patients with viral illnesses such as mumps and measles (Haire, 1972). In order to map the gene region which is responsible for anti-actin antibody production, I studied the immune response to actin in C57Bl/10 mice and the B10 congenic mice. Strains B10.BR, B10.AKM and B10.A(4R), which carry k alleles at the K locus in the class I MHC region, produced anti-actin antibodies when immunized with actin alone, whereas the other strains which do not carry the k alleles at the K locus, C57Bl/10, B10.D2, B10.A(5R), A.TH, and A.TL, did not produce anti-actin antibodies. C57Bl/10 mice were identified as being low responders and the congenic B10.BR as being high responders when immunized with native actin (Table 4.2). On the other hand, lymph node T cells from both C57Bl/10 and B10.BR immunized mice responded to actin stimulation. T cells from the high responder mice (B10.BR) had a higher response than the T cells from lower responder mice (C57Bl/10) (Fig. 4.1). This result implies that actin specific T cells are activated after actin immunization. The activities of these T helper cells were blocked by monoclonal antibodies to class II-MHC antigens (Fig. 4.2 A).

Effect of M protein on the immune response to actin. When mice were immunized with a mixture of actin and M protein, all strains of mice produced a high titer of anti-actin antibodies (Table 4.2). The anti-actin antibody production is no longer restricted to the k alleles at the K locus in the class I MHC regions. Lymph node T cells from actin plus M protein immunized mice were used to study the responses to actin, and M protein. T cell responses to actin and M protein were observed in C57Bl/10 and B10.BR mice. Both actin and M protein specific T helper cells from low responder mice (C57Bl/10) showed lower responses to the antigens compare to the responses from high responder mice (B10.BR). Furthermore, the T cell proliferation to actin, M protein and PPD were decreased by the addition of monoclonal antibodies to class II MHC antigens (Fig. 4.2 B). However, monoclonal antibodies to class II MHC antigen did not affect the T cell response to concanavalin A (con A), which is a known T cell mitogen.

Carrier effect of M protein on anti-actin antibody production. Groups of five C57Bl/10 mice were immunized with M protein, actin mixed with M protein (A+M), keyhole lympets hemocyanin (KLH) or actin covalently linked with KLH (A-KLH) one month before they were received a booster injection. The results showed that there was no immune response to actin in M protein or KLH primed mice which were boosted with A+M or A-KLH (Fig. 4.3). When mice

were immunized with A+M or A-KLH, all animals produced anti-actin antibodies but mice immunized with actin and boosted with actin did not produced anti-actin antibodies. Therefore, the interaction of actin and M protein or KLH is essential for the induction of anti-actin antibody production.

DISCUSSION

Many viruses, such as EBV (Kurki and Virtanen, 1984), measles, mumps (Haire, 1972) and influenza virus (Portocala *et al.*, 1988), can trigger autoimmune responses by one of several proposed mechanisms. In Chapter 3, I studied the effect of paramyxovirus membrane (M) protein on the immune response to actin as a model of virus-induced autoimmunity. M protein enhanced the anti-actin antibody production in rabbits and mice. The augmentation in mice was not a result of molecular mimicry since there is no cross-reactivity of anti-actin antibodies to M protein or vice versa. The immune response to actin is controlled by immune response genes (H-2 genes), since MHC congenic mice show different T-cell responses (Fig. 4.1) and differences in the ability to produce antibodies to actin (Table 4.2). B10.BR, B10.AKM and B10.A(4R) mice, which have k alleles at the K locus in the class I region, are defined as high responders since they produced antibody to native actin. The other congenic strains, C57Bl/10, B10.D2, B10.A(5R) are defined as low responders because they did not produce anti-actin antibodies when immunized with native actin. Since it was difficult to obtain congenic B10 mice which carried K allele in the class II region but other alleles in the class I region, I had to confirm the results seen in B10 mice in other strains of congenic mice. A.TH and A.TL mice differ only at the class II locus; *i.e.* A.TH carries the s allele whereas A.TL carries

the k allele. Both failed to respond to native actin. On the basis of these observations in two congenic strains (B10 and A), it appears that the production of antibodies to native actin is controlled by MHC class I antigen. Therefore, the T cells that help in the production of anti-actin antibodies must be class-I restricted. However class II-restricted helper T cells may also be induced during this process. However, these class I-restricted T cells which provide help for the anti-actin antibody production may express class II antigens on the cell surface since their proliferation was abolished by monoclonal antibodies to class II-MHC antigens (Fig.4.2 A).

B10.BR mice and C57Bl/10 mice have the same genetic background except at the MHC region. As shown in Fig. 4.1, it was found that T cells from both strains could be activated by actin. However, T cells from B10.BR mice gave higher responses at comparable antigen doses. The actin specific T cells in low responder mice does not appear to provide significant help for anti-actin antibody production (Table 4.2). One possible reason could be that class I-restricted T helper cells may be required for anti-actin antibodies. Such cells are only induced in B10.BR mice while C57Bl/10 mice may allow induction of class II-restricted T helper cells.

The effect of paramyxovirus M protein on anti-actin antibody production in several strains of mice was examined.

All strains, both high responders and low responders, produced higher titers of anti-actin antibodies when they were immunized with actin mixed with M protein (Table 4.2). There appears to be no cross-reactivity of M protein and actin at the level of the T cell response because T cells from actin-immunized mice did not respond to M protein *in vitro* (Fig. 4.2, panel A). The T cell responsivenesses to either actin or M protein in low responder mice were lower than in high responder mice (Fig. 4.1 C,D). This may account for immunological tolerance to actin in low responder mice. Furthermore, the actin specific T cells and M protein specific T cells in actin plus M protein immune mice are class II-restricted since their activities were blocked by monoclonal antibodies to class II MHC antigens. However, class II bearing class I-restricted T cells discussed above may also be present in these T cells.

The induction of anti-actin antibody production is not due to M protein specific helper T cell activities since M protein immune mice did not produce anti-actin antibodies when they were challenged with actin plus M protein (Fig. 4.3). Milich *et al.*(1987) demonstrated that antibody production to the nucleocapsid and envelop proteins of the hepatitis B virus can be induced by immunization with the nucleocapsid protein. Even though the envelop and nucleocapsid antigens are found on separate molecules, the results showed that helper T cell primed with a synthetic T

cell epitope of nucleocapsid protein can induce B cells to produce antibodies that react with several epitopes on the envelop antigen. A similar approach has been used to demonstrate whether M protein or keyhole limpets hemocyanin (KLH), a well known carrier, can help B cells to produce antibodies to actin. The results showed that only mice primed with actin mixed with M protein or actin covalently linked to KLH produced anti-actin antibodies whereas mice immunized with M protein or KLH alone did not produce anti-actin antibodies. These data imply that M protein or KLH specific T cells can not enhance B cells to produce antibodies to actin. Furthermore, either crosslinking of actin to KLH or mixing of actin with M protein is sufficient to induce the anti-actin antibody production. Therefore, a mechanism other than carrier effect or molecular mimicry must account for the augmentation of anti-actin antibodies as a result of the mixing of actin with M protein.

Previous studies in our laboratory have proved that there was an interaction of actin and M protein. Evidences from the circular dichroism studies of the actin-M protein complex show that there was an increase of 3% in α-helix, with asssociated decrease of 1 and 2% in β-structure and random coil from the predicted spectrum of actin-M protein complex (Giuffre *et al.*, 1982). These changes may alter the processing of actin by antigen-presenting cells. Altered processing may lead to a different response to the antigen.

Altered processing may overcome the class I-restriction of antibody response. Our results support this hypothesis since even the non-responders to native actin will produce anti-actin antibodies when immunized with actin plus M protein. While the response to native actin appears to be class I-restricted, the response to actin plus M protein is not.

Although the immunodominant epitopes on native actin are class I-restricted, actin might also carry hidden epitopes which are not class I-restricted. As shown in Table 4.2, the anti-actin antibody titers of B10.BR mice immunized with actin alone are lower than the titers in animals immunized with actin plus M protein. The lack of antibody production in the low responder mice can be explained by an inefficiency in antigen processing. Support for the theory of altered processing by APCs comes from studies using native or denatured myoglobin. Processing of the unfolded, denatured form of myoglobin known as S-methyl myoglobin reveals some new antigenic sites which were not identified when native myoglobin was used as as an immunogen (Berzofsky *et al.*, 1988). Furthermore, hindering structures in the flanking region of an antigenic fragment left after the processing of native myoglobin may interfere with binding of this fragment to the MHC molecule or to the T-cell receptor. In the denatured S-methyl myoglobin, the antigenic sites are produced without the hindering flanking regions, therefore the epitope is more easily expressed with MHC class II and

can better interact with T-helper cells (Brett *et al.*, 1988). By analogy, the binding of M protein may change the tertiary structure of actin in such a way that the hidden epitopes are processed or presented by the antigen presenting cells to the T-helper cells more efficiently. The presentation of these new antigenic epitopes is less stringently restricted since all mouse strains can produce a response to actin in the presence of M protein.

In conclusion, the immune response to actin is influenced by genetic and environmental factors. The anti-actin antibody production to native actin is restricted to class I-MHC gene products. These class I-restricted helper T cell may also carry class II-MHC antigens on the cell surface. Paramyxovirus M protein primed T cells did not help B cells to produce antibody to actin. However, M protein induces conformational changes in actin to either reveal new B cell epitopes or to alter the processing of actin in order to trigger an autoimmune response. If M protein reveals B cell epitopes on actin, the actin-M protein complex itself may be able to activate B cells to produce anti-actin antibodies without the need for T cells. On the other hand, the change in antigen processing could cause a switch in the response from a class I to a class II response; alternatively, it render immunogenic peptides more readily presentable to T cells in conjunction with class II-MHC molecules. This may serve as a model for the mechanism for a

virally induced alteration in the response to a host cell protein and a subsequent autoimmune response.

TABLE 4.1*

Properties of Class I and Class II MHC Molecules.

	Class I	Class II
Genetic loci	H-2K, H-2D, H-2L in mice; HLA-A, HLA-B, HLA-C in humans	I-A and I-E clusters in mice; DP, PQ, DR, and one or two other clusters in humans
Chain structure	α chain (~45,000 daltons) +β2 microglobulin (11,500 daltons)	α chain (29,000 to 34,000 daltons) +β chain (25,000 to 28,000 daltons)
Cell distribution	almost all nucleated cells	B cells, antigen-presenting cells, thymus epithelial cells, some others
Involved in presenting antigen mainly to	cytotoxic T cells	helper T cells
Polymorphic domains involved in T cell recognition and antigen binding	α1+α2	α1+β1

* Alberts *et al.*, 1989.

TABLE 4.2

Genetic control of immune response to actin using actin primed and actin mixed with M protein-primed mice

Mouse strain	Haplotype of H-2 subregions				$\log_{10}$ antibody titer in mice primed with	
	K	I-A	I-E	D	Actin	Actin+M protein
C57B1/10J	b	b	b*	b	-	3.408
B10.D2	d	d	d	d	-	2.806
B10.BR	k	k	k	k	2.20	3.408
B10.AKM	k	k	k	q	1.60	3.408
B10.A(4R)	k	k	b*	b	1.60	2.806
B10.A(5R)	b	b	k	d	-	3.408
A.TL	s	k	k	d	-	3.408
A.TH	s	s	s*	d	-	3.408

*I-E molecules in mouse haplotype b and s are not expressed because of the E gene mutation. (Klein, 1986).

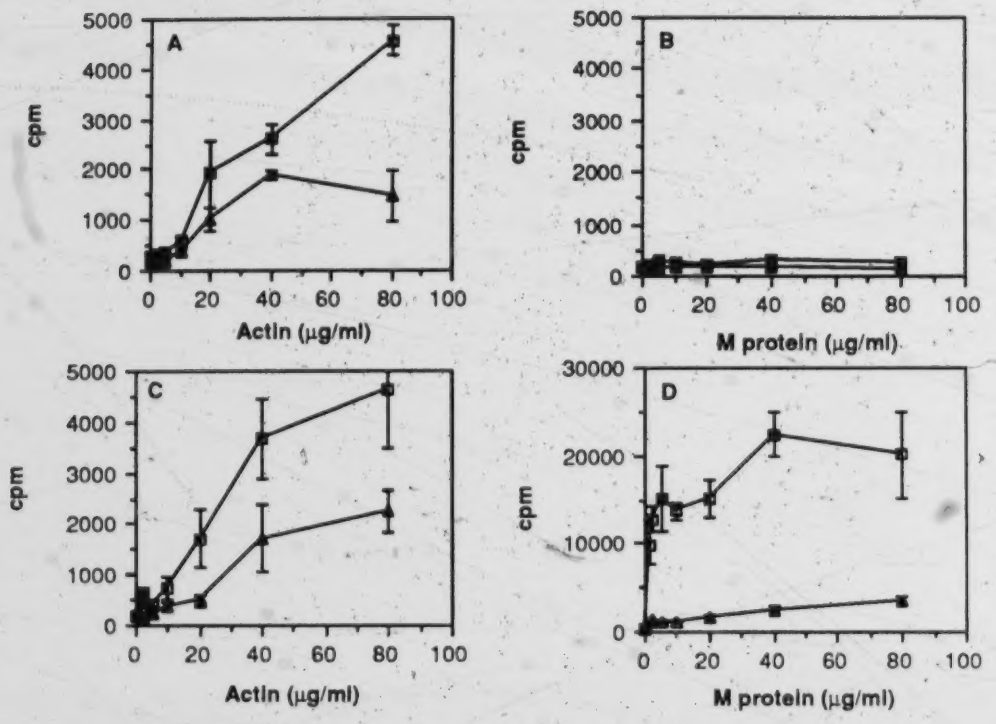


Figure 4.1 Comparative studies of immune response to actin from T cells of B10.BR mice (□) and C57Bl/10 mice (Δ) in actin immunized mice (panel A and panel B) and actin plus M protein immunized mice (panel C and panel D). The responses were measured by ^{3}H-thymidine incorporation into T cells at different concentrations of actin in T cell proliferation assays.

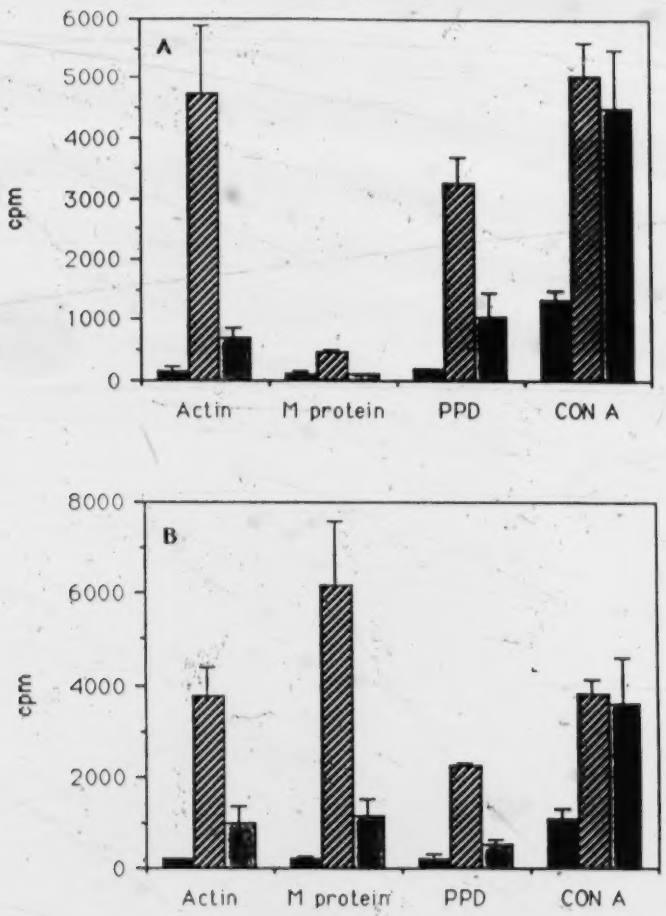


Figure 4.2 Effect of monoclonal antibodies to MHC antigen on T cell proliferation stimulated by actin, M protein, PPD and con A. B10.BR lymphocyte culture from actin immunized mice (panel A) and actin plus M protein immunized mice (panel B) were treated with antibodies to class II antigens and cultured in 80 µg/ml actin, 40 µg/ml M protein, 20 µg/ml PPD, or 5 µg/ml con A. These microcultures were assayed according to the T cell proliferation procedure as described in Materials and Methods.

■ ^{3}H-thymidine uptake in the absence of T cells.(APCs only)

▨ ^{3}H-thymidine uptake of T cells and APCs.

■ ^{3}H-thymidine uptake of T cells and APCs in the presence of monoclonal antibodies to class II antigens.

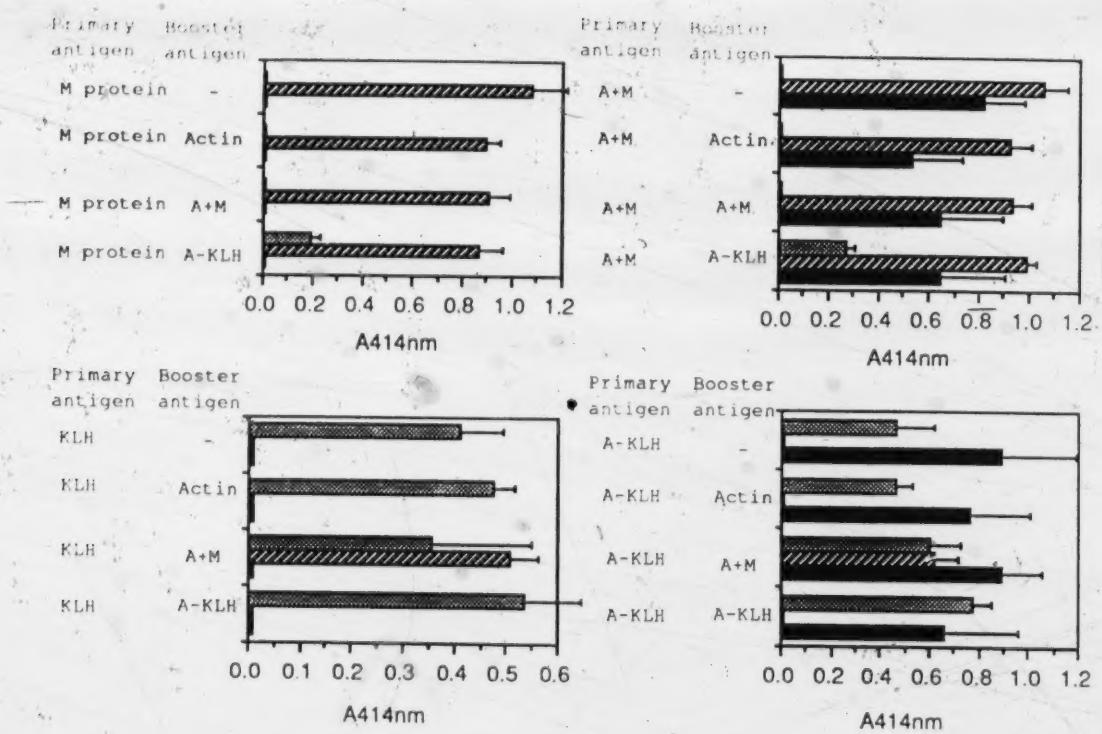


Figure 4.3 Carrier priming effect on anti-actin antibody production. C57B1/10 mice were immunized with M protein, actin mixed with M protein (A+M), KLH, or actin covalently linked with KLH (A-KLH). They were received booster injection 1 month after the primary immunization. Mouse antisera were taken 10 days after the booster injection and they were used for assaying antibodies to actin (■), M protein (▨) and KLH (■) by ELISA. Mice immunized with actin alone and boosted with actin under the same condition did not produced anti-actin antibodies.

BIBLIOGRAPHY

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. 1989. Molecular biology of the cells. 2nd edition. Garland Publishing. Inc. New York pp. 1042.

Berzofsky, J.A., Brett, S.J., Streicher, H.Z., and Takahashi, H. 1988. Antigen processing for presentation to T lymphocytes: function, mechanisms and implication for the T-cell repertoire. Immunol. Reviews. 106: 5-31.

Braciale, T.J., Morrison, L.A., Sweetser, M.T., Sambrook, J., Gething, M-J., and Braciale, V.L. 1987. Antigen presentation pathways to class I and class II MHC-restricted T lymphocytes. Immunol. Reviews. 98: 95-114.

Brett, S.J., Cease, K.B., and Berzofsky, J.A. 1988. Influences of antigen processing on the expression of the T-cell repertoire: Evidence for MHC-specific hindering structures on the products of processing. J. Exp. Med. 168: 357-373.

Cafruny, W. A., and Hovinen, D.E. 1988. Infection of mice with lactate dehydrogenase-elevating virus leads to stimulation of autoantibodies. J. Gen. Virol. 69: 723-729.

Cafruny, W.A., Heruth, O., Jaqua, M.J., and Plagemann, P.G.W. 1986. Immunoglobulins that bind to uncoated ELISA plate surfaces: appearance in mice during infection with Lactate dehydrogenase-elevating virus and in human anti-nuclear antibody positive sera. J. Med. Virol. 19: 175-186.

Eaton, M.D. 1980. Autoimmunity induced by syngeneic membranes carrying irreversibly absorbed paramyxovirus. Infect. Immun. 27: 855-861.

Fujinami, R. S., and Oldstone, M.B.A. 1985. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: Mechanism for autoimmunity. Science. 230:1043-1045.

Germain, R.N. 1986. The ins and outs of antigen processing and presentation. Nature. 322: 687-689.

Haire, M. 1972. Fibrillar anti-cellular antibody associated with mumps and measles infection. Clin. exp. Immunol. 12: 335-341.

Klein, J. 1986. Natural history of the major histocompatibility complex. pp. 216. John Wiley & Sons, Inc. USA.

Krah, D. L., and Choppin, P.W. 1988. Mice immunized with measles virus develop antibodies to a cell surface receptor for binding virus. J. Virol. 62(5):1565-1572.

Kurki, P., and Virtanen, I. 1984. The detection of human antibodies against cytoskeletal components. J. Immunol. Method. 67: 209-213.

Lee, K. C., Singh, B., Barton, M.A., Procyshyn, A., and Wong, M.. 1979. A simple reliable system for studying antigen-specific murine T cell proliferation. J. Immunol. Methods. 25: 159-170.

Lippincott-Schwartz, J., Yuan, L.C., Bonifacino, J.S., and Klausner, R.D. 1989. Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. Cell. 56: 801-813.

Milich, D.R., McLachlan, A., Thronton, G.B., and Hughes, J.L. 1987. Antibody production to the nucleocapsid and envelop of the hepatitis B virus primed by a single synthetic T cell site. Nature. 329: 547-549.

Moore, M.W., Carbone, F.R. and Bevan, M.J. 1988. Introduction of soluble protein into the class I pathway of antigen processing and presentation. Cell 54: 777-785.

Morrison , L.A., Lukacher, A.E., Braciale, V.L., Fan, D.P., and Braciale, T.J. 1986. Differences in antigen presentation to MHC class I- and class II- restricted influenza virus-specific cytolytic T lymphocyte clones. J. Exp. Med. 163: 903-921.

Murray, J.S., Madri, J., Tite, J., Carding, S.R., and Bottomly, K. 1989. MHC control of $CD4^+$ T cell subset activation. J. Exp. Med. 170: 2135-2140.

Nepom, J.T., Weiner, H. L., Dichter, M.A., Tardieu, M., Spriggs, D.R., Gramm, C.F., Powers, M. L., Fields, B. N., and Green, M.I. 1982. Identification of a hemagglutinin-specific idiotype associated with reovirus recognition shared by lymphoid and neural cells. J. Exp. Med. 155: 155-167.

Nuchtern, J.G., Biddison, W.E., and Klausner, R.D. 1990. Class II MHC molecules can use the endogenous pathway of antigen presentation. Nature 343: 74-76.

Oldstone, M.B.A. 1987. Molecular mimicry and autoimmune disease. Cell. 50: 819-820.

Oldstone, M.B.A., and Notkins, A.L. 1986. Molecular mimicry. In "Concepts in viral pathogenesis II", Oldstone, M.B.A., and Notkins, A.L. (eds.), pp. 195-209. Springer-Verlag, New York.

Perkins, D.L., Lai, M-Z., Smith, J.A., and Gefter, M.L. 1989. Identical peptides recognized by MHC classI- and II-restricted T cells. J. Exp. Med. 170: 279-289.

Portocala, R., N. Spyrou, V. Lambropoulord, E. Pateraki. 1988. The presence of both antivirus and antiself antibodies in sera from patients with adenovirus and influenza B. Virologie. 39(3): 207-216.

Scheid, A., and Choppin, R.W. 1973. Isolation and purification of envelope protein of Newcastle disease virus. J. Virol. 11:263-271.

Spudich, J. A., and Watt, S. 1971. The regulation of rabbit skeletal muscle contraction. J. Biol. Chem. 246:4866-4871.

Townsend, A., and Bodmer, H. 1989. Antigen recognition by class I-restricted T lymphocytes. Ann. Rev. Immunol. 7: 601-624.

CHAPTER 5

Effect of the Expression of the Cloned M Gene on Actin Microfilaments in Fibroblasts

Paramyxoviruses assemble at the plasma membrane of infected cells and produce infectious virus by budding (Dubois-Dalcq *et al.*, 1984). It has been found that the newly synthesized polypeptides of the transcriptive complex rapidly associate with the cytoskeletal framework and are assembled with 50 S viral RNA into nucleocapsids which remain bound to the cytoskeletal framework (Hamaguchi *et al.*, 1985). The actual mechanism of the virus budding process is unknown. A recent comprehensive review on paramyxovirus M protein outlines the central role of M protein in the process of viral budding (Peeples, 1990). The absence of functional M protein is believed to account for the lack of budding of viral particles in subacute sclerosing panencephalitis (SSPE) infected brain cells (Hall and Choppin, 1979; ter Meulen *et al.*, 1983). M protein forms a bridge between the viral glycoproteins on the infected cell membrane and the nucleocapsid in the cytoplasm (Choppin and Compans, 1975). It can assemble into ordered arrays *in vitro*. This supports the concept proposed by Yashida *et al.* (1976) that ordered arrays of M protein are involved in the assembly of the virus particles at the cell membrane (Heggeness *et al.*, 1982).

A role for actin microfilaments in the budding process of some viruses such as measles virus (Tyrrell and Ehrnst, 1979; Bohn *et al.*, 1983; and Stallcup *et al.*, 1983) and NDV (Morrison and McGinnes, 1985) has been proposed. Morphological evidence for the role of actin in virus budding has been presented in the electron microscopic studies of Bohn *et al.*, (1986). The number of actin filaments was decreased in NDV, Sendai and measles virus-infected cells (Fagraeus *et al.*, 1981). Biochemical studies have shown that actin forms a complex with virus M protein and there was a conformational change in actin as a result of this interaction (Guiffre *et al.*, 1982). The association of paramyxovirus M protein and cellular actin may account for the observation that actin is a structural component in many viruses, including paramyxoviruses. The decrease in number or disruption of actin microfilaments in paramyxovirus infected cells may be a result of M protein interaction with actin. To study the effect of M protein on the actin microfilaments, I have cloned the gene for measles virus M protein into a eukaryotic expression vector and expressed M protein in fibroblast cell lines.

MATERIALS AND METHODS

Plasmid DNA

pMSG, a eukaryotic expression vector, was purchased from Pharmacia. Measles virus M gene clone (pF1-MVMX) was a generous gift from Dr. W. Bellini (Center for Disease Control, Atlanta, Georgia). This is a full length M gene clone in the riboprobe vector pSP64 between the SalI and PstI restriction sites. The SP6 polymerase will produce genomic sense RNA. This clone includes a sequence of 30 nucleotides of the P gene 3' end and the entire reading frame of the M plus the 450 non-coding nucleotides at the 3' end of the M message.

Preparation of HB101 competent cells

The *E. coli* strain of HB101 competent cells was prepared according to the method of Sambrook *et al.* (1989). Ten microliters of frozen stock were incubated in 5 ml LB broth at 37 °C overnight. One ml of the overnight culture was added to 200 ml of LB broth and incubated in a shaker incubator at 37 °C for 3-5 h until the absorbance of culture at 550 nm was 0.4-0.6. The bacterial cells were harvested by centrifugation at 5,000 rpm for 10 min at 4 °C. The pellet was resuspended in 100 ml of ice-cold 50 mM $CaCl_2$ in 10 mM Tris-Cl, 1 mM EDTA, pH 8.0 (TE buffer). It was put on ice for

15 min and centrifuged at 5000 rpm for 10 min at 4 °C. The pellet was resuspended in 8.5 ml of ice-cold 50 mM $CaCl_2$ in TE buffer and 15% glycerol. It was aliquoted into 200 µl samples and stored at -80 °C until used in transformation.

Transformation of HB101 competent cells

Two hundred microliters of frozen competent cells were thawed on ice and incubated with 10 ng of plasmid DNA for 20 min. The bacterial cells were recovered by centrifugation at 12,000 g for 30 seconds and resuspended in 1 ml of warm LB medium. The suspension was incubated at 37 °C for 45 min to allow the bacterial cells to grow. The cells were harvested by centrifugation at 12,000 g for 30 seconds and resuspended in 100 µl of LB medium. The culture was plated on LB agar containing 100 µg/ml ampicillin and incubated at 37 °C overnight. The ampicillin-resistant colonies were picked and propagated for the preparation of plasmid DNA.

Small scale preparation of plasmid DNA by alkaline lysis

The minipreparation of plasmid DNA has been previously described by Sambrook *et al.* (1989). A single bacterial colony on LB agar plate containing 100 µg/ml ampicillin was transferred into 5 ml LB medium containing ampicillin (100 µg/ml) in a snap-capped 15 ml tube. The culture was incubated overnight at 37 °C. A 1.5 ml portion of the overnight culture was transferred to a 1.5 ml microfuge tube

and centrifuged at 12,000 g for 30 seconds in a microfuge. The bacterial pellet was resuspended in 100 µl 50 mM glucose, 25 mM Tris-Cl (pH 8.0) and 10 mM EDTA containing 4 mg lysozyme/ml. The solution was incubated at room temperature for 10 min. Then 200 µl of freshly prepared 0.2 N NaOH and 1% SDS were added and the contents were mixed by inverting the tube rapidly. The tube was stored on ice for 10 min. One hundred fifty microliters of ice cold 3 M potassium acetate was added to the contents, which were mixed and held on ice for 5 min. The pellet was removed by centrifugation for 5 min at 12,000 g in a microfuge. The supernatent was transferred to a new tube and extracted with an equal volume of phenol:chloroform. The double-stranded DNA in the aqueous solution was precipitated with 2 volumes of ethanol at room temperature. After 5 min, the pellet was recovered by centrifugation and washed with 1 ml cold 80% ethanol and dried under vacuum. The DNA sample was dissolved in 50 µl of TE buffer, pH 8.0 containing 20 µg DNase free pancreatic RNaseA/ml and incubated at room temperature for 20 min before being stored at -20 °C. An aliquot of the DNA preparation was analyzed for purity on agarose gel electrophoresis.

Subcloning pMVM DNA into a eucaryotic expression vector (pMSG)

The pMSG plasmid DNA was completely digested with SalI enzyme and the DNA fragment was purified by 0.8% agarose gel electrophoresis as described by Sambrook *et al.* (1989).

The pMVM cDNA was obtained by digestion of pF1-MVMX DNA with restriction endonucleases PstI and SalI. Separation and recovery of the cDNA copy of the PstI-SalI fragment containing the M gene was carried out by electrophoresis in 0.8% agarose gel followed by trapping of the appropriate DNA band onto DE81 cellulose paper and elution with 1 M NaCl in TE buffer. Two volumes of cold ethanol was added to the eluted DNA and the sample was incubated at -20 °C for 1 h. The pellet was recovered, washed with 1 ml 80% ethanol, resuspended in an appropriate volume of TE buffer and quantitated by fluorometry (Morgan *et al.*, 1979).

The PstI-SalI fragment of pMVM cDNA was incubated with T4 DNA polymerase in 15 µl of its buffer (from BRL) at room temperature for 15 min in order to fill in the ends. The DNA solution was extracted with one volume of phenol:chloroform followed by a second extraction with an equal volume of chloroform. The aqueous phase was spin-dialysed on a millipore membrane against TE buffer (200 ml) for 20 min. The purified DNA fragment (10 µl) was ligated to SalI linker by overnight incubation with T4 DNA ligase in 20 µl ligation buffer (0.5 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM dithiothreital and 1 mM ATP) at 14 °C. The DNA solution was extracted with an equal volume phenol:chloroform followed by a second extraction with an equal volume of chloroform. The aqueous phase was spin-dialysed on millipore membrane against 200 ml TE buffer for 20 min. The dialysed DNA solution was

incubated with the linear pMSG SalI fragment in the presence of T4 DNA ligase and its buffer at 14 °C overnight. The product was analyzed by electrophoresis in a 0.8% agarose gel. The bacteria *E. coli*, strain HB101 was used for transformation by the ligated plasmid DNA. Recombinant clones which carried the pMVM insert DNA were identified by small scale preparation of plasmid DNA and confirmation by electrophoresis. Plasmid pMR DNA carries the proper coding orientation of the inserted M gene, whereas pMW DNA carries the reverse orientation.

Large scale preparation of plasmid DNA by cesium chloride (CsCl) gradient centrifugation

DNA to be transfected into fibroblast cell lines was purified by CsCl gradient centrifugation as described by Sambrook *et al.* (1989). Two ml of an overnight culture grown from a single colony were inoculated into 400 ml of LB medium containing 100 µg/ml amipicillin in a 2-liter flask which was incubated overnight on a rotary shaker at 37 °C with vigorous shaking (300 cycles/minute). The overnight cultures were harvested by centrifugation at 4000 rpm for 15 min at 4 °C. The pellets were resuspended in 18 ml lysozyme buffer (50 mM glucose, 25 mM Tris-Cl, pH 8.0 and 10 mM EDTA). Two ml of freshly prepared lysozyme solution (10 mg/ml in lysozyme buffer) was added and the preparation was incubated at room temperature. After a 10 min incubation, 40 ml of 0.2 N NaOH in 10% SDS was added to the solution which was kept on ice

for 10 min. Twenty ml of ice-cold 3M potassium phosphate was added and the solution was kept on ice for 10 min before centrifugation at 4000 rpm for 15 min at 4 °C. The supernatants were transferred into 250-ml centrifuge bottles followed by the addition of 0.6 volumes of isopropanol, mixed well by gently swirling, and stored at room temperature for 15 min. The nucleic acids were recovered by centrifugation at 10,000 rpm for 20 min at 4 °C. The pellets were washed with 70% ethanol and allowed to dry overnight. The dry pellets were resuspended in 2.6 ml of TE buffer and 4.0 g of solid CsCl was added with gentle mixing until the salt was dissolved. The final volume was approximately 3.8 ml. Ethidium bromide (0.2 ml of a 10 mg/ml solution) was added to the nucleic acid solution which was stored in the dark until used.

A CsCl solution (ρ=1.47 g/ml) was prepared by adding 29 g solid CsCl to 35 ml of TE buffer. Eight ml of CsCl solution was put into a Beckman Quick-Seal centrifuge tube and the nucleic acid-ethidium bromide solution was placed under the CsCl using a 9-inch Pasteur pipette. If necessary, the tubes were filled with the CsCl solution before being sealed. The gradients were centrifuged at 65,000 rpm at 20 °C for 5 h. The closed circular plasmid DNA, which was the lowest visible band, was collected and the ethidium bromide was removed from the DNA by extraction with n-butanol saturated with water. The DNA solution was then dialysed

against 4.0 liters of TE buffer overnight at 4 °C to remove excess salt. Following this, a 1/10 volume of 3 M sodium acetate pH 7.5 and 2 volumes cold ethanol were added and the solution was incubated at -20 °C for 1 h. The pellet was recovered, washed with 1 ml 80% ethanol and resuspended in 1 ml of TE buffer and quantitated by fluorometry (Morgan *et al.*, 1979).

Transfection of fibroblast cells with recombinant DNA

Two methods of transfection were attempted in these studies.

a) Transfection by calcium phosphate precipitation

CV-1 (a monkey kidney cell line) and NIH 3T3 (

a mouse fibroblast cell line) cells were purchased from the American Type Culture Collection (ATCC). They were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing penicillin, streptomycin, 25 mM HEPES pH 7.3, and 10% fetal calf serum. Twenty hours after seeding approximately 10^6 cells on 100-mm plates, the cultures were transfected. On the day of transfection, cells were fed with 9.0 ml of complete medium 2 to 4 h prior to addition of the precipitate containing $CaPO_4$ and DNA. Transfection was performed with 10-20 µg of either pMR or pMW using the calcium phosphate precipitation method as described by Gorman (1985). In brief, the DNA-$CaPO_4$ precipitate was prepared as

follows: 240 µl of distilled water containing 10 µg DNA was mixed with 240 µl of 0.5 M $CaCl_2$ in 0.1 M HEPES buffer pH 7.12. The solution was incubated for 10 min at room temperature. Then it was mixed with 480 µl of 0.28 M NaCl, 0.05 M HEPES, 0.75 M NaH_2PO_4, and 0.75 M Na_2HPO_4, pH 7.12. The precipitate was incubated for 15 min at room temperature before being added to the cells. Four hours after transfection, the cells were washed twice with sterile PBS and shocked with 2 ml of 15% glycerol in isotonic HEPES buffer, pH 7.5, for 3 min at room temperature. Cells were rinsed twice with medium without any supplements and then were fed with complete medium until being harvested.

b) Transfection of DNA by electroporation

Cells were cultured until approximately 90% confluent. Five to six million cells were harvested from the tissue culture plate and washed with Dulbecco Modified Eagle's Medium without any supplements, resuspended in 0.8 ml in the same medium with 20 µg purified plasmid DNA. The cells were electroporated at 250 volts and 950 microfaradays capacitance using a "gene pulser" (BIORAD). Cells were stored on ice for 10 min before dispersing and culturing in the complete medium for 24 h. The culture were fed with 10 ml of fresh medium and incubated for 2 more days.

Transient expression vs. stable expression

For transient expression, cells were harvested seventy-two hours after transfection since the transgenome lack of functional centromeres and the transfected gene expressed a sharp peak at 48-72 h post-transfection. Cells were stained using indirect immunofluorescent labelling to detect the transfected gene product. For stable expression, cells were fed with selection medium containing 10% dialyzed fetal calf serum, xanthine (250 µg/ml), hypoxanthine (15 µg/ml), thymidine (10 µg/ml), aminopterin (2 µg/ml) and mycophenolic acid (25 µg/ml). Mycophenolic acid and aminopterin will select for transfected cells which expressed the *E.coli gpt* gene. After the first twenty-four hours, the culture fluid was replaced with fresh selection medium containing the same supplements and this medium change was repeated every 3 days thereafter. After 10 days, the selectively transformed cells were cultured in complete medium and used in the immunofluorescence studies.

Double labelled immunofluorescence technique to detect the expression of M protein and its effect on actin microfilaments

Cells which were grown on the slides were washed by dipping in PBS three times. The slides were fixed with cold acetone at -20 °C for 20 min, air dried and kept at -20 °C until used in the immunofluorescence studies.

The fixed slides were warmed to room temperature and incubated with 1:20 phalloidin-conjugated rhodamine for 20 min at 37 °C. Phalloidin has been shown to bind to actin microfilaments (Faulstich *et al.*, 1988). After staining with phalloidin, the cells were washed in PBS three times. The cells were then incubated with polyclonal anti-measles virus antibodies (a gift from Dr. R. Marusyk), diluted 1 in 200 in 1% BSA, for 40 min at 37 °C. They were washed three times in PBS followed by incubation with 1:70 fluorescein-conjugated goat anti-rabbit antibodies (FITC) for 30 min at 37 °C. The cells were washed three times in PBS and mounted with 90% glycerol in PBS. The cells were examined by fluoresence microscopy with appropriate filters in order to study the morphology of actin filaments in the presence and absence of M protein expression.

RESULTS

Construction of transfection vectors

The initial construct used in this study was the plasmid pMSG, which is composed of the ampicillin resistance gene and the origin of replication from plasmid pBR322 joined to a hybrid eukaryotic transcription unit.[3] The plasmid pMSG is designed for inducible expression of cloned genes in mammalian cells. Its transcriptional unit is composed of the glucocorticoid inducible promotor present in the mouse mammary tumor virus long terminal repeat (MMTV LTR), the selectable marker *E. coli* xanthine guanine phosphoribosyltransferase (gpt) gene which is expressed from simian virus 40 (SV40) early promoter, and SV40 mRNA processing signals including the early splice region and polyadenylation site. The PstI-SalI segment of cloned measles virus M gene from pFL-MVMX plasmid was inserted into the SalI site of the multiple cloning site located at the 3' end of the MMTV LTR region. The insert is translated from the first AUG codon and its expression is sensitive to hormonal induction. Plasmid pMR is the construct with the normal coding orientation of the M gene insert, whereas pMW is the antisense orientation (Fig. 5.1). Three out of eight colonies carried the inserted gene (Fig. 5.2). One colony carried the antisense M gene construct and two colonies carried the M gene in the desired (coding) orientation (Fig.

5.3). Each type was used in the transfection of fibroblast cell lines.

Transfection and selection of the transformed cells

Fibroblast cell monolayers were transfected with the recombinant plasmid DNA and were selected based on the fact that the *E. coli* gene (*Ecogpt*) that encodes xanthine-guanine phosphoribosyltransferase (XGPRT) is analogous to the mammalian enzyme hypoxanthine phosphoribosyltransferase. Both enzymes catalyze the conversion of hypoxanthine and guanine to IMP and GMP, respectively. The bacterial enzyme also converts xanthine to XMP, a step which is poorly catalyzed by the mammalian enzyme. Thus, *E. coli* XGPT can provide the purine salvage function of mammalian HPRT when the mammalian enzyme is inhibited by mycophenolic acid and aminopterin and cells are grown selectively in the hypoxanthine/aminopterin/thymidine medium. The selection relies on the fact that *Ecogpt*-transformed cells, but not normal cells, can use xanthine to overcome an inhibition of the *de novo* synthesis of GMP (Fig. 5.4).

Measles virus M gene expression in NIH 3T3 cells

NIH 3T3 cells were transfected with either pMR, pMW, or pCH110 (a eukaryotic expression vector carrying the bacterial *lacZ* gene which expresses the β-galactosidase enzyme). Cells transfected with pCH110 produced the enzymes which catalyzed the production of p-nitrophenol, whereas the non-transfected

cells did not. The enzyme activities were assayed by the method previously described (Sambrook *et al.*, 1989).

Comparative studies using immunofluorescent staining of the pMR and pMW transfected NIH 3T3 cells showed that cells transfected with pMR expressed M protein which disrupted actin microfilaments (Fig. 5.6) whereas cells without M protein expression still had intact microfilaments. This result is consistent with the observation that measles virus infection disrupts the actin microfilaments in measles virus infected-CV-1 cells (Fig. 5.5). The disruption of microfilaments appeared to be dependent on the level of measles virus M protein expression in the infected cells. This was difficult to quantify.

DISCUSSION

Several lines of evidence have implied an interaction of cytoskeleton and viral protein during the viral assembly and budding processes. An extensive alteration of the structure of actin filaments has been observed in BHK 21 cells infected with Newcastle disease virus (NDV) or vesicular stomatitis virus (VSV) (Rutter and Mannweiler, 1977); in herpes simplex virus-infected BHK 21 cells (Bibor-Hardy *et al.*, 1985); in measles, mumps, Sendai virus-infected human fibroblast cells (Fagraeus *et al.*, 1978) and in vaccinia virus-infected mouse 3T3 cells (Meyer *et al.*, 1981).

Network formation by actin filaments is inhibited by cytochalasins (MacLean-Fletcher and Pollard, 1980). Use of these drugs to study the effect of microfilaments on virus production has yielded conflicting results. Cytochalasin B has been reported to be effective in blocking the production of virus particles in VSV- or influenza virus-infected cells (Griffin and Compans, 1979). However, these authors felt that the effect of cytochalasin B was a result of blocking virus shedding through inhibition of glycosylation rather than an effect on actin microfilaments. Morrison and McGinnes (1985) found that cytochalasin D accelerated the release of NDV from infected cells. This result implies that the cellular cytoskeleton functions to slow the release of virus particles from cells. Other authors have found

evidence for a positive role of actin microfilaments in virus budding. Stallcup *et al.* (1983) have provided convincing evidence for a role of actin microfilaments in the budding process of measles virions. Bohn *et al.* (1986) provided morphological evidence for the role of microfilaments in the intracellular transport of viral proteins and the budding of measles virus using electron microscopy. These results agree with the studies of Tyrrell and Ehrnst (1979) which demonstrated a physical link between surface glycoproteins and intracellular measles nucleocapsid through actin microfilaments.

Paramyxovirus M protein, which is known to play a central role in virus maturation, has been shown to interact with cellular actin (Giuffre *et al.*, 1982). Furthermore, experiments with cloned influenza virus M protein in a vaccinia virus vector and its expression in infected cells demonstrated the intracellular co-localization of viral M protein and actin (Bucher *et al.*, 1989). In order to study the interaction of viral M protein with the cytoskeleton without using cytochalasins which have some undesirable effects on the cells, I have expressed cloned measles virus M protein in fibroblast cell lines. The results show that the actin microfilament network in cells expressing M protein is disrupted (Fig. 5.6). This observation would support the hypothesis that it is M protein that interacts with and is responsible for the disruption of actin microfilaments in

measles-infected cells. These experiments are not ideal due to the rather high unexpected background staining of NIH 3T3 cells by my measles antisera. This was not seen with Vero or CV-1 cells. Further studies using measles antisera preabsorbed with uninfected NIH 3T3 cells are being done.

Before I can unequivocally conclude that the disruption of actin microfilaments in measles virus-infected cells is a result of M protein interaction with actin, it would be necessary to have a negative control with N protein expression in NIH 3T3 cells. Unfortunately, time does not permit me to complete this control experiment.

During the time I was planning the experiments in this chapter, Bucher *et al.* (1989) published their findings on the expression of influenza M protein in CV-1 cells. These studies show an interaction of M protein with actin microfilaments, a finding which agrees with mine. In addition, Blondel *et al.* (1990) studied the role of M protein in the cytopathogenesis of vesicular stomatitis virus (VSV) using a temperature sensitive (ts) mutant of the M protein of VSV. This mutant fails to cause morphological change of infected cells at the non-permissive temperature (40 °C). However, at the permissive temperature (37 °C) it will cause cytopathic changes. These authors cloned the gene for ts mutant of VSV M protein and transfected this clone into Cos cells. It was difficult to detect transfected cells at the permissive temperature because the expression of M protein

caused disruption of cytoskeleton and rounding up of the cells. These cells became detached. However at the non-permissive temperature, the ts mutant was expressed but remained perinuclear and did not disrupt the cytoskeleton or cause a cytopathic effect. Since my studies were not using a ts mutant expression, it will cause a cytopathic effect and the cells would become detached. This may explain some of the difficulty I encountered in trying to demonstrate transformed cells.

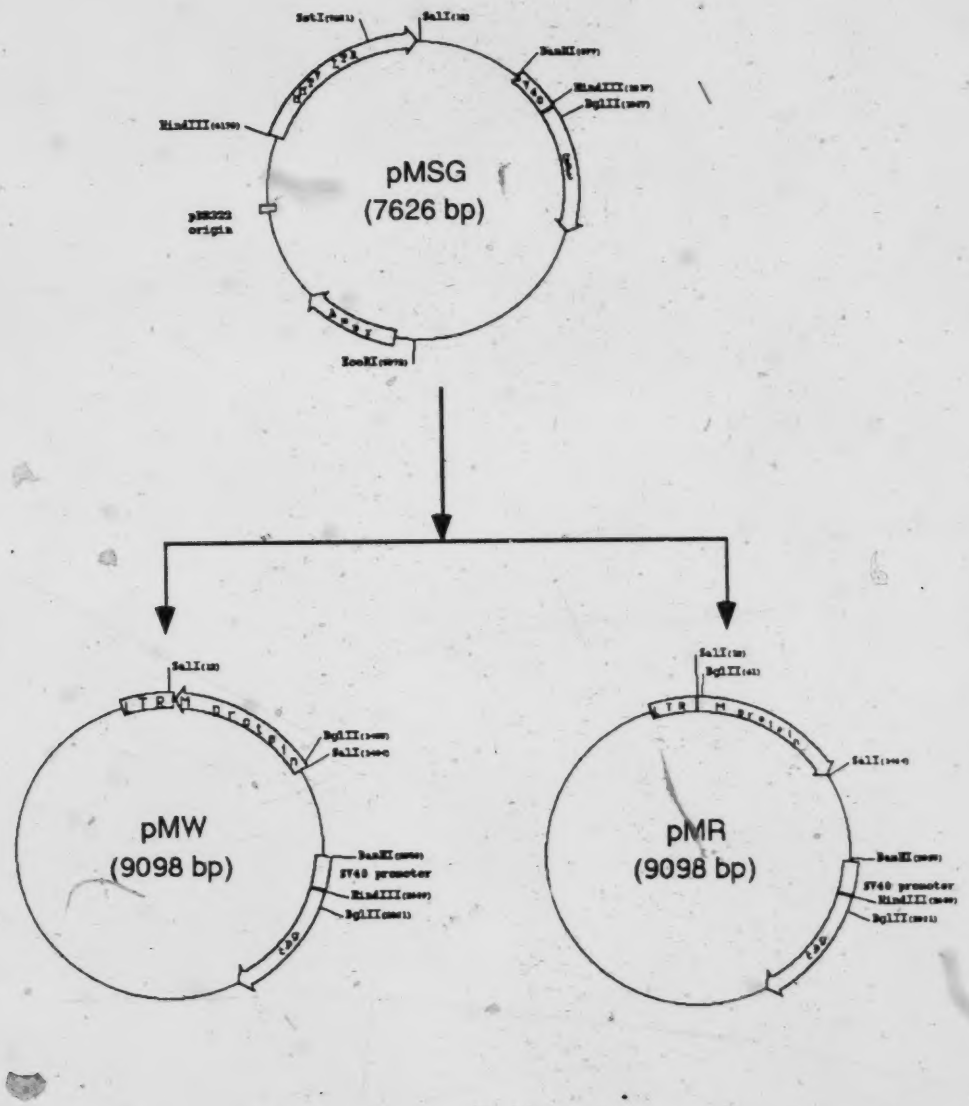


Figure 5.1 Genetic maps of plasmid DNA used in this study. The cloned measles viral M gene was inserted into a eukaryotic expression vector (pMSG) with the desired orientation (pMR) and the reverse orientation (pMW).

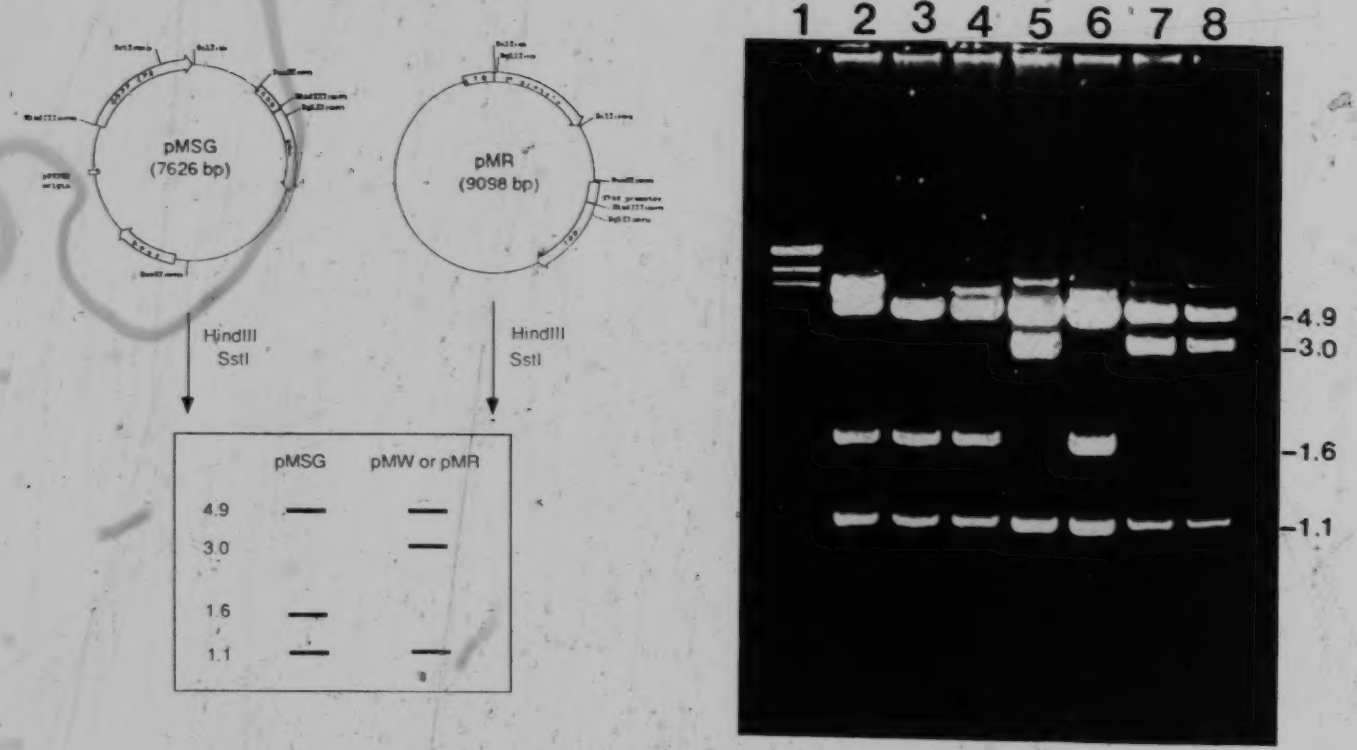
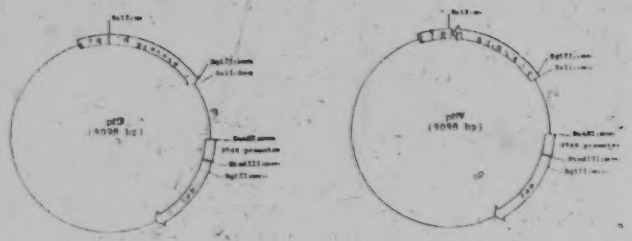
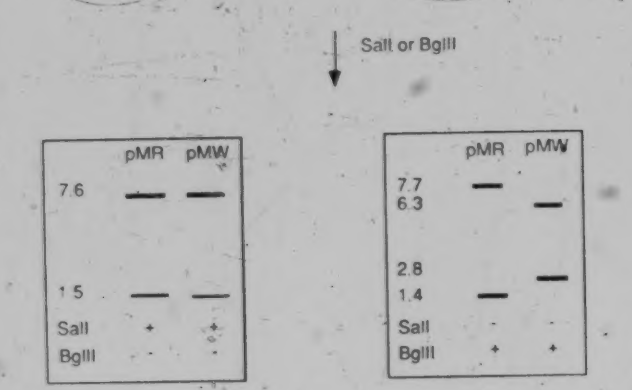


Figure 5.2 Agarose gel electrophoresis of plasmid DNA digested with restriction endonucleases, HindIII and SstI to confirm the insertion of cloned M gene. Lane 1 is the λ DNA digested with HindIII. Lane 2-8 are the recombinant DNAs isolated from the transformed cells and the DNAs were digested with HindIII and SstI.





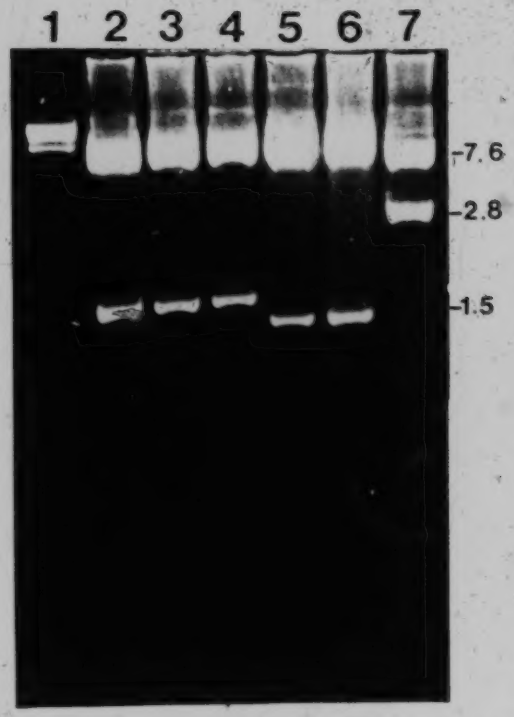


Figure 5.3 Plasmid DNA digested with restriction endonucleases, SalI or BglII. The digestion of recombinant DNA with SalI gave 7.6 and 1.5 kb fragments. Digestion with BglII is used to demonstrate the orientation of the insert DNA. Lane 1 is the λ DNA digested with HindIII. Lane 2-4 are the recombinant DNAs digested with SalI. Lane 5-7 are the recombinant DNAs digested with BglII.

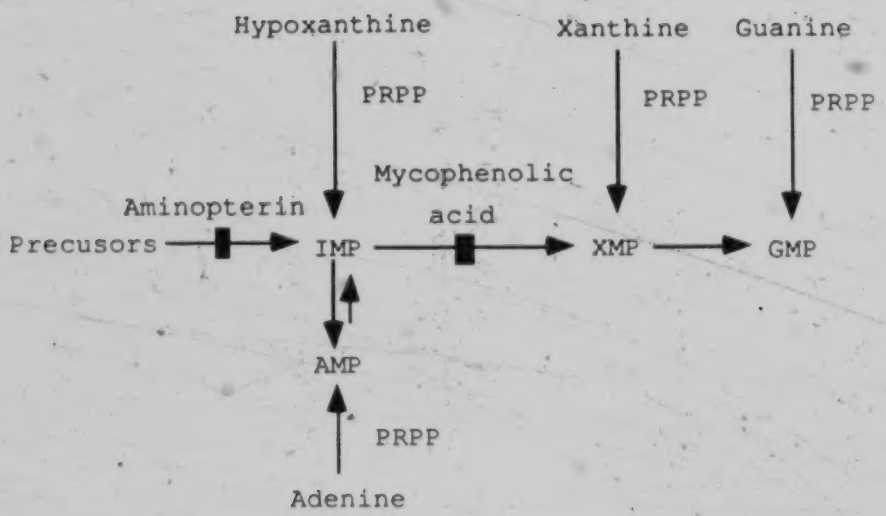


Figure 5.4 Pathway of purine nucleotide synthesis and the inhibition sites of aminopterin and mycophenolic acid.

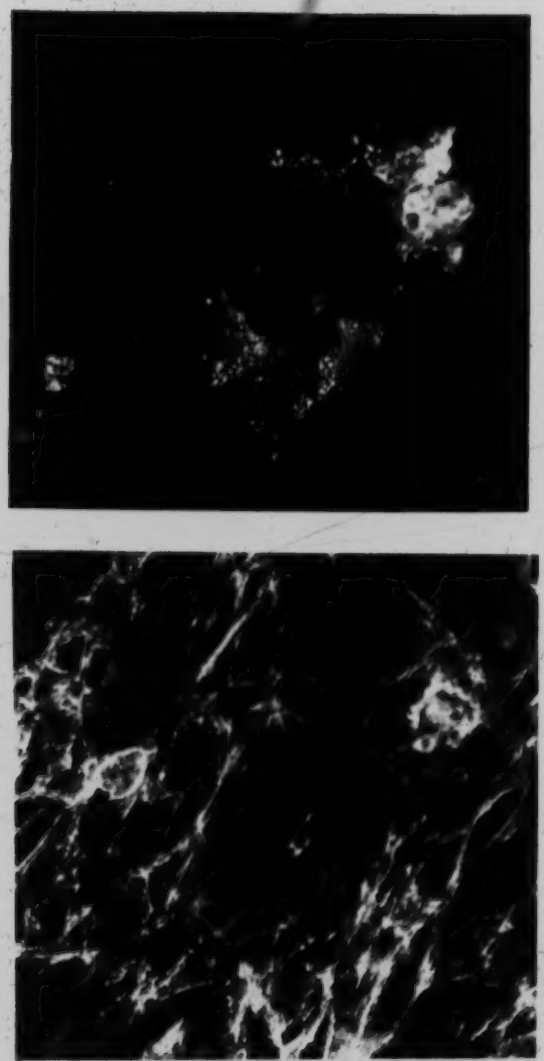


Figure 5.5 Double labelled immunofluorescent staining of measles virus-infected CV-1 cells. Cells in the upper panel were stained with polyclonal antibodies to measles virus. Cells in the lower panel were stained with rhodamine conjugated phalloidin to demonstrate the actin microfilaments. CV-1 cells were infected with lec strain measles virus at MOI = 1 and incubated at 37 °C for 24-48 h before being stained.

Figure 5.6 Double labelled immunofluorescent staining of pMR transfected NIH3T3 cells. Cells in the upper panel were stained with polyclonal antibodies to measles virus. Cells in the lower panel were stained with rhodamine conjugated phalloidin to demonstrate the actin microfilaments. NIH 3T3 cells were transfected with pMR DNA by electroporation at 250 V, 960 microfaradays and transferred to complete medium containing 5% fetal calf serum and 0.1 μM dexamethasone. They were incubated at 5% CO_2, 37 °C for 48 h before being fixed and stained.

BIBLIOGRAPHY

Bibor-Hardy, V., Bernard, M., and Simard, R. 1985. Nuclear matrix modifications at different stages of infection by herpes simplex virus type 1. J. Gen. Virol. 66: 1095-1103.

Blondel, D., Harmison, G.G., and Schubert, M. 1990. Role of matrix protein in cytopathogenesis of vesicular stomatitis virus. J. Virol. 64: 1716-1725.

Bohn, W., Rutter, G., Hohenberg, H., and Mannweiler, K. 1983. Inhibition of Measles virus budding by phenothiazines. Virology. 130:44-55.

Bohn, W., Rutter, G., Hohenberg, H., Mannweiler, K., and Nobis, P. 1986. Involvement of actin filaments in budding of measles virus: studies on cytoskeleton of infected cells. Virology. 149(1): 91-106.

Bucher, D., Popple, M., Baer, M., Mikhail, A., Gong, Y.F., Whitaker, C., Paoletti, E., and Judd, A. 1989. M protein (M1) of influenza virus: antigenic analysis and intracellular localization with monoclonal antibodies. J. Virol. 63(9): 3622-3633.

Choppin, P.W., and Compans, R.W. 1975. Reproduction of paramyxoviruses. In "Comprehensive virology", Fraenkel-Conrat, H., and Wagner, R.R. (eds.), vol. 4, pp. 95-178, Plenum Press, New York, New York..

Fagraeus, A., Tyrrell, D.L.J., and Norrby, E. 1978. Actin filaments in paramyxovirus-infected human fibroblasts studied by indirect immunofluorescence. Arch. Virol. 57: 291-296.

Faulstich, H., Zobeley, S., Rinnerthaler, G., and Small, J.V. 1988. Fluorescent phallotoxins as probes for filamentous actin. J. Muscle Res. and Cell Motility (British Journal) 9: 370-383.

Giuffre, R.M., Tovell, D.R., Kay, C.M., and Tyrrell, D.L.J. 1982. Evidence for an interaction between membrane protein of a paramyxovirus and actin. J. Virol. 42: 963-968.

Gorman, C. 1985. High efficiency gene transfer into mammalian cells. In "DNA cloning Volume II, a practical approach", Glover, D.M. (ed.), pp. 143-190.

Hall, W.W., and Choppin, P.W. 1979. Evidence for lack of synthesis of the M polypeptide of measles virus in brain

cells in subacute sclerosing panencephalitis. Virology. 99:443-447.

Hamaguchi, M., Nishikawa, K., Toyoda, T., Yoshida, T., Hanaichi, T., and Nagai, Y., 1985. Transcriptive complex of Newcastle disease virus. Virology. 147: 295-308.

Heggeness, M. Smith, P. and Choppin, P. 1982. *In vitro* assembly of the nonglycosylated membrane protein (M) of Sendai virus. Proc. Natl. Acad. Sci. U.S.A. 75: 6232-6236.

MacLean-Fletcher, S., and Pollard, T.D. 1980. Mechamism of action of cytochalasin B on actin. Cell. 20: 329-341.

Meyer, R.K., Burger, M.M. Tschannen, R., and Schafer, R. 1981. Actin filament bundles in vaccinia virus infected fibroblasts. Arch. Virol. 67: 11-18.

Morgan, A.R., Lee, J.S., Pulleyblank, D.E., Murray, N.L., and Evans, D.H. 1979 Review: Ethidium fluorescence assays. Part 1. Physicochemical studies. Nucleic Acids Research. 7(3): 547-570.

Morrison, T.G., and McGinnes, L.T. 1985. Cytochalasin D accelerates the release of Newcastle disease virus from infected cells. Virus. Res. 4: 93-106.

Peeples, M.E. 1990. Paramyxovirus M proteins: pulling it all together and taking it on the road. In "The paramyxoviruses", Kinsbury, D.W. (ed.), Plenum press. (in press).

Rutter, G., and Mannweiler, K. 1977. Alteration of actin-containing structures in BHK 21 cells infected with Newcastle disease virus and vesicular stomatitis virus. J.Gen.Virol. 37: 233-242.

Sambrook, J., Fritsch, E.F., Maniatis, T. 1989. Molecular cloning: a laboratory manual. 2nd. edition. Cold Spring Harbor Laboratory Press.

Stallcup, K.C., Raine, C.S., and Fields, B.N. 1983. Cytochalasin B inhibits the maturation of measles virus. Virology. 124(1): 59-74.

ter Muelen, V., Stephenson, J.R., and Kreth, H.W. 1983. Subacute sclerosing panencephalitis. In "Comprehensive virology", vol. 18, Frankel-Conrat, H., and Wagner, R.R. (eds.), pp.105-159. New York, Plenum press.

Tyrrell, D.L.J., and Ehrnst, A. 1979. Transmembrane communication in cells chronically infected with measles virus. J. Cell Biology 81: 396-402.

CHAPTER 6

General Conclusions and Future Prospects

Genetic factors which influence anti-actin antibody production were investigated. Studies on the production of anti-actin antibodies in B10 congenic mice immunized with native actin showed that the antibody response was controlled by class I-MHC genes. Only mice with the k allele at the class I MHC locus produced antibodies against native actin. Changing the haplotypes from the k allele to other alleles at the D locus (one of the two class I loci) reduced the antibody production to native actin, as shown in Table 4.2 for B10.BR, B10.AKM, and B10.A(4R) mice. When both class I loci were lacked the k allele (C57Bl/10, B10.D2, B10.A(5R) mice), the animals produce no anti-actin antibidies in response to immunization with native actin. By comparison, all mice immunized with an actin-M protein complex produced higher titers of anti-actin antibodies. These results support the concept that the immunodominant epitopes on native actin (primarily in the N-terminal region) are class I-restricted, whereas some of the cryptic epitopes which are revealed upon the interaction with viral M protein are class II-restricted.

I used the monoclonal antibodies to K^k (class I-antigens) and I-A^k (class II-antigens) to block the T cell response to actin, M protein, and PPD. I thought this would differentiate class I and class II-restrictions. To my surprise, the results indicated that antibodies to both class I and class II antigens blocked the T cell responses to all of these soluble antigens. In general, immune responses to soluble antigens such as PPD are class II-restricted. The cross-blocking effect of the monoclonal antibodies to class-I and class II antigens might be the result of the close proximity of class I and class II molecules on the surface of the APCs. More insight into the control of this response could be carried out by using reagents such as chloroquine and the fungal antibiotic brefeldin-A (BFA) to pretreat antigen presenting cells (APCs). The class I-restricted responses should be more sensitive to BFA than to chloroquine whereas the class II-restricted responses should be more sensitive to chloroquine than BFA (Nuchtern *et al.*, 1990).

The interaction between a specific viral protein, paramyxovirus M protein, and a specific cellular protein, actin, which results in the production of anti-actin antibodies serves as a model for a mechanism of virus-induced autoimmunity. To elucidate the structural and functional relationships involved in this interaction, I have used both biochemical and immunological approaches. The physical association of these two proteins shows a high affinity on

the basis of the equilibrium binding assays. The dissociation constant of 6.3×10^{-8} M or binding affinity of 1.6×10^{7} M^{-1} is considered to be strong binding activity. This binding constant falls in the same range as those of other actin binding proteins (Table 2.1). This is also in the same range as many antibody-antigen interactions; for example, antibodies to staphylococcal nuclease have an affinity constant of 8.3×10^{8} M^{-1} (Sachs *et al.*, 1972)

M protein binds to actin at a site different from the DNaseI binding site. This might be the reason that the binding of M protein to actin enhances the antibody production to actin whereas the binding of DNaseI does not. In contrast, the binding of M protein does not augment the production of antibodies to carbonic anhydrase in mice. However, the enhancement effect of M protein on the production of antibodies to carbonic anhydrase may be more difficult to demonstrate since carbonic anhydrase is an immunogenic protein.

This specific augmentation of antibody production to actin has been demonstrated in mice and rabbits. Our studies also show that human sera from patients recovering from measles virus have anti-actin antibodies. The results from my immunoblotting studies indicate that the interaction of viral M protein with actin enhances antibody production to new antigenic epitopes on the actin molecule. These new sites are located between amino acid residues 40-113, 114-226

and 227-375. This result supports the concept that a viral protein can alter a host cell protein and make it more antigenic to the immune system, possibly through a conformational change.

Further studies demonstrating the exposure of new epitopes as a result of a conformational change seem warranted. Mejean *et al.* (1988) have demonstrated a cation-induced conformational change of actin using an immunological approach. A series of antibodies reacting with amino acid sequences 1-7, 18-28, 40-113, 168-226 and 285-375 were used as surface probes to study the structural relationship between antigenic epitopes and cation binding to actin. The antibodies were used in an ELISA to monitor the antigenic activity of G- and F-actins in the presence of Ca^{+2} or Mg^{+2}. A similar approach could be applied to study the effect of M protein on actin. The experiments could be done by using antibodies raised against amino acid sequences 1-7, 18-28, 40-113 and 168-226. The strength of the antibody binding to actin could serve as a monitor of possible conformational change as a result of the interaction of M protein with actin. These antibodies may all bind readily to the actin-M protein complex but not so readily to native actin. This would support the concept that binding to M protein would result in making some antigenic site on actin more accessible to antibody molecule.

The coating of monomeric or filamentous actin on the ELISA plates has no significant effect on their conformation (Mejean *et al.*, 1988). Therefore, immobilized actin on ELISA plates can be used for the preadsorption of antiserum from animals immunized with actin alone or actin-M protein complexes. The adsorbed sera could be used in Western blotting studies. Preadsorption with actin may selectively remove only antibodies to the N-terminus (1-62) whereas adsorption with actin-M protein complexes may remove antibodies to all epitopes including the "new" epitopes. These types of adsorption studies may confirm the exposure of "new" epitopes as a result of conformational changes in actin after interaction with M protein.

Another approach to demonstrate the class I restriction of the immune response to actin could be pursued through studies of the T-cell responses in congenic mice in order to map genes controlling the response to actin, T cells from mice immunized with actin alone, or actin-M protein complex will be used. The study of T-cell response in different strains of congenic mice should yield a more straightforward answer than using monoclonal antibodies to MHC antigens to selectively block the T-cell responses.

One may ask how altered conformation may lead to an augmented immune response. This may result from altered processing by APCs. Brett *et al.* (1988) reported that

altering the conformation of antigen in the flanking region surrounding the antigenic epitopes can affect the processing of APCs and lead to changing of MHC restriction. The new individual T cell clones which were induced by these "newly processed antigenic peptides" were identified in the antigen immune-T cell population. However, these T cell clones did not have a pronounced effect on the corresponding antigenic peptides when the T cell proliferation assays were performed by using the antigen immune-T cell population. A similar line of reasoning may explain the non-enhancing effect of the actin-M protein complex on the T cell response, but an enhancing effect on the antibody production to actin. That is the "new T helper cells" which do not show a significant effect on T cell response can provide help to B cells for anti-actin antibody production. Future research to support this hypothesis could be done by using immunogenic peptides to sensitize animals and to select the T cell clones from the actin-M protein complex-immune T cell population which are responsive to these peptides. However, the antigenic mapping on actin molecules in the actin-M protein complex must be completed first. I believe that some antigenic peptides will be presented by class I-MHC molecules whereas other antigenic peptides will be presented by class II-MHC molecules. The antigenic sites which responded in the animals immunized with native actin alone would be class I-restricted whereas the others which resulted from the interaction of actin and M protein would be class II-restricted.

In conclusion, this system seems to be a useful model to study virus-induced autoimmunity. Although the mechanisms have not been conclusively proven, some intriguing possibilities have emerged. These include: a) the binding of M protein to actin changes the tertiary structure of actin to make some antigenic sites more accessible to the immune system; b) the interaction of M protein with actin appears to alter its processing by antigen presenting cells; c) the interaction of M protein with actin results in more efficient T help for the antibody response to weak antigenic epitopes, or d) the binding to a viral protein results in the switching of the antibody production to a host cell protein from a class I to a class II immune response. More studies with this system should provide more insight into the mechanism of some viral-induced autoimmune responses.

BIBLIOGRAPHY

Brett, S.J., Cease, K.B., and Berzofsky, J.A. 1988. Influences of antigen processing on the expresssion of the T-cell repertoire: Evidence for MHC-specific hindering structures on the products of processing. J. Exp. Med. 168: 357-373.

Mejean, C., Hue, H.K., Pons, F., Roustan, C., and Benyamin, Y. 1988. Cation binding sites on actin: a structural relationship between antigenic epitopes and cation exchange. Biochem. Biophys. Res. Commun. 152(1): 368-375.

Nuchtern, J.G., Biddison, W.E., and Klausner, R.D. 1990. Class II MHC molecules can use the endogenous pathway of antigen presentation. Nature 343: 74-76.

Sachs, D.H., Schechter, A.N., Eastalke, A., and Anfinsen, C.B. 1972. Inactivation of staphylococcal nuclease by the binding of antibodies to a distinct antigenic determinant. Biochemistry. 11: 4268-4273.